REMARKS

The requisite fee for a three-month extension of time can be charged to Deposit Account No. 02-1818. Any fees that may be due in connection with filing this paper or with this application during its entire pendency may be charged to Deposit Account No. 02-1818. If a Petition for extension of time is required, this paper is to be considered such Petition, and any fee charged to Deposit Account No. 02-1818.

A Supplemental Information Disclosure Statement is filed on the same day herewith, under separate cover.

Claims 50-52, 73- 79, 81, 84, 87-95, 97-99, 101, 111, 114-115, 117, 119-121, 128 are pending. Claim 92 is amended for clarity. No new matter is added.

The Examiner states that all claim objections and rejections of record not specifically addressed below are considered hereby withdrawn in response to Applicant's arguments. The remaining rejections for lack or written description and enablement are premised on the allegation that the specification does not teach or describe the generation of **plant SATACs**. These rejections have been addressed in previous Actions and Responses thereto of record and such Responses and accompanying documents are incorporated by reference. Applicant's responses below rebut the Examiner's arguments.

REBUTTAL TO EXAMINER'S "RESPONSE TO PRELIMINARY ARGUMENTS"

1) The Examiner states that:

> ***The state of the art has been established in prior office actions and the state of the art suggests that it would not have been merely routine in the art to apply the teachings regarding mammalian chromosomes to plant chromosomes.... the claims are not limited to only generating SATACs de novo as urged by Applicant. The claims are drawn to introducing SATACs into a plant cell via any method taught in the specification, including whole, in vitro assembled SATACs. Therefore the claims will remain rejected for the reasons of record as the rejections still apply to the broadly drawn claims. In response to applicant's argument that the references fail to show certain features of applicant's invention, it is noted that the features upon which applicant relies (i.e., de novo centromere formation) are not recited in the rejected claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See In re Van Geuns, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).***

Applicant understands that the Examiner is interpreting the claims in their broadest manner. As noted by the Examiner, however, the claims must at least be interpreted in view of all claim limitations and in light of the specification. Thus, there has to be some

"reasoned" interpretation of the subject matter of the claims, and the claims should be interpreted in their broadest "reasonable interpretation". MPEP 2111 states:

> During patent examination, the pending claims must be "given their broadest reasonable interpretation consistent with the specification." [citing *Phillips v. AWH Corp.*, 415 F.3d 1303, 75 USPQ2d 1321 (Fed. Cir. 2005)]

Further, MPEP 2111.01 states that:

> This means that the words of the claim must be given their plain meaning unless the plan meaning is inconsistent with the specification.... An applicant is entitled to be his or her own lexicographer and may rebut the presumption that claim terms are to be given their ordinary and customary meaning by clearly setting forth a definition of the term that is different from its ordinary and customary meaning(s).

This is particularly apt in this case, where the term satellite artificial chromosome (SATAC) is introduced by the Applicant to describe the artificial chromosomes discovered by Applicant, and which heretofore had not been known and could not have had a name. Applicant is permitted to be his/her own lexicographer; the application is directed to SATACs among several types of artificial chromosome. The specification must be reviewed in order to interpret the claims. It is not sufficient for the Examiner to merely state that the broadest interpretation includes an *in vitro* assembled SATAC because the Examiner wants to rely on the state of the art that at the time of filing the application to show that the claims are not enabled. In this case, the claims specify that a SATAC contains more heterochromatin then euchromatin and the specification renders it clear that a SATAC does not encompass an *in vitro* artificial chromosome assembled from component parts.

SATACs are defined and described and depicted in the specification. SATACs are defined and described as being a distinct type of artificial chromosome compared to an *in vitro* synthesized artificial chromosome, see e.g. at page 15, lines 1-4, which states that among the artificial chromosomes provided are SATACs, minichromosomes, and *in vitro* synthesized artificial chromosomes. Thus, a SATAC is clearly a distinct type of artificial chromosome that is characterized by containing more heterochromatin then euchromatin and additional defining features. SATACs are defined as having more heterochromatin then euchromatin and include sets of tandem DNA blocks each containing satellite DNA flanked by non-satellite DNA. A SATAC includes inverted repeats of satellite DNA and the heterologous DNA, which occurs by virtue of the discovery of processes for producing SATACs (see e.g. at page 18, lines 24-26; at page 19, lines 22-24). The beauty of the SATAC is that the cell produces it when a DNA fragment is introduced into the cell and the

cell is cultured as described in the application. No knowledge of centromere structure or any structure is needed. The DNA fragment integrates into the pericentric heterochromatin, either by targeted integration, or, just by random integration. Upon integration into that region a series of amplification events that include *de novo* formation of a second centromere (not from the DNA fragment, but by the chromosome duplicating the existing centromere). The amplification events also produce the repeating DNA blocks and ultimately produce sausage chromosomes and other structures that result in SATACs. For the *in vitro* assembled artificial chromosomes as described in the specification, the SATACs can serve as a source of centromeres or other components for such *in vitro* assembly, but SATACS are **not** *in vitro* assembled artificial chromosomes.

A reading of the specification renders it clear that a SATAC does not encompass an *in vitro* generated artificial chromosome. For example, the specification states that SATACs can be used as the starting materials for assembling chromosomes *in vitro* by serving as a source for the identification and isolation of components to be used in *in vitro* constructed artificial chromosomes, i.e. the centromere and origin of replication. Thus, it is clear that a SATAC itself is not an *in vitro* constructed chromosome. Further, the specification states that the method of *de novo* generation of satellite artificial chromosomes overcomes problems in reconstructing artificial chromosomes *in vitro* (see *e.g.* at page 4, lines 6-25) because one does not have to produce a centromere or any components of the SATAC. SATACs are generated by the cell. Hence, the specification provides not only methods of *de novo* generation of mammalian artificial chromosomes, but based on the universal discovery, also provides "methods for constructing species-specific artificial chromosomes *de novo*" (see *e.g.* at page 5, lines 6-7).

Notwithstanding this, the standard for enablement does not require a teaching of all ways to practice the claimed subject matter, nor that all ways have to work well. The standard for enablement is just that the specification teaches the claimed subject matter such that one of skill in the art, without undue experimentation, can make and use the subject matter as claimed. The Examiner is reminded that not all embodiments within the scope of a claim must be operative:

> [I]t is not a function of the claims to specifically exclude either possible inoperative substances or ineffective reacting proportions". In Application of Dinh-Nguyen, 492 F.2d 865 at 858-9 181 USPQ 46 (CCPA (1974)).

The specification teaches how to make SATACs in great detail. Whether or not a SATAC could be assembled by assembly of the component parts does not mitigate enablement because the specification teaches how to make them in cells. The DECLARATIONS of record demonstrate the generation of SATACs in plants based on the teachings of the specification. These Declarations unequivocally rebuts any argument that given the state of the art one would not be able to generate SATACs in plants. As the DECLARATIONs state, all that had to be done was follow the teachings of the specification by introducing a DNA fragment into a plant cell and "poof" SATACs were generated. The Examiner must give deference to the DECLARATIONS, unless they can show that one of skill in the art would have a rationale basis to doubt the truth of such statements.

The Examiner cannot base rejections on cited references, and ignore the teachings of the specification and the DECLARATIONS of record. The prior art does not benefit from the teachings of the instant specification. None of the references partake in the benefit of the teachings of the specification, which describes a method that overcomes limitations of generating artificial chromosomes discussed in the references. The instant method do not require knowledge of chromosomal structures or sequences. None of the references make such assertions regarding generation of SATACs using the method set forth in the specification and claims (e.g. claim 115 and 128), in any species, including in plants, and the resultant SATACs or intermediates or precursors, use of these references to establish the state of the art is inapt. Furthermore, none partake of benefit of the teachings of the instant application, which is a significant factor to be considered. The Examiner has provided no rationale basis based on the cited references for doubting the teachings of the specification for the generation of SATACs.

2) The Examiner states that:

Applicants urge that centromeric sequences are not necessary to identify SATACs…. There is no guidance in the specification that indicates the recognition of the centromere and thus, the SATAC in a plant cell.

As stated previously, it is not necessary to have knowledge of, nor to identify, the centromere to generate or identify a SATAC. The DECLARATIONs did not require or use any knowledge of a plant centromeric sequence. DNA fragments were introduced into plant cells and "poof" SATACs resulted. SATACs contain a centromere because they are made by the cells, which results in *de novo* formation of the centromere. It respectfully is submitted that since the method occurs *de novo* upon introduction of a DNA fragment into a

cell, resulting in the *de novo* formation of a centromere, no knowledge of the centromere base sequence, or of the position of the replication origin is required. The method described in the specification overcomes problems of generating artificial chromosomes in any species because all that is required is to introduce a DNA fragment into a cell and grow the cells under selective conditions, such that the amplification event can be observed. This was so stated in the specification. For example, at page 4, line 9 to page 5, line 5, the specification states that:

> ...construction of defined mammalian artificial chromosomes has not been achieved. Such construction has been hindered by the lack of an isolated, functional, mammalian centromere and uncertainty regarding the requisites for its production and stable replication...Thus, development of alternative artificial chromosomes ...is required....It is a further object herein to provide methods for constructing species-specific artificial chromosomes *de novo*.

The specification teaches that SATAC's are characterized by other unique features such as the existence of large-scale amplification of integrated DNA and heterochromatin, the presence of more heterochromatin then euchromatin interspersed with heterologous DNA, and its existence as a stable extragenomic chromosomal structure (see *e.g.* at page 5, lines 14-19; at page 16, line 22 to page 17, line 4; at page 18, line 24-26; at page 19, lines 4-24; at page 27, l;ines 18-21; at page 34, line 23; at page 35, lines 12-15; at page 62, lines 5-7). The specification teaches and describes that a sausage chromosome is a chromosome containing amplified heterochromatin and a euchromatic arm (see *e.g.* at page 18, line 21-24). Based on these features, SATACs and sausage chromosomes can be identified.

The application describes these structural features, which have been discussed in great detail in previous responses. For example, Figures 2F (panel 2-8) and the bottom structure depicted in Figure 3 each depict the identifying characteristics of a SATAC. The bottom panels of each of Figures 2 and 3 depict a distinct chromosome structure that is predominately heterochromatin. It also depicts a structure that is interspersed with foreign DNA that has been integrated into the de novo formed chromosome based on homologous recombination and amplification events that occur during the formation of the SATAC. Like the application, the DECLARATIONS of record identify SATACs based on these structural features. As taught and described in the specification, such features can be identified using such methods as Southern hybridization, *in situ* hybridization, including fluorescence *in situ* hybridization, and G and C-banding. (see below under heading 4) for rebuttal to Examiner's

comments that these techniques do NOT work in plants, as they indeed do and have been so-known for many years).

For example, the specification describes the use of Southern Blot to identify the copy number of the integrated DNA sequence to evidence its amplification. The existence of multiple copies of the integrated DNA (*e.g.* containing a selectable marker) is indicative of the amplification methods that result by practice of the method. Accordingly, that contain SATACs and other amplified structures with the repeating regions of satellite DNA flanked by heterologous DNA can be identified, such as by analyzing cells by Southern hybridization for those that contain multiple copies of the integrated exogenous DNA (see *e.g.*, at page 31, lines 31 to page 32, line 3; at page 107, line 25 to page 108, line 4). This is further exemplified in the DECLARATIONS. For example, DECLARATION 5 and 7 demonstrates by Southern blot that the selectable marker is present at greater than 20 copies, evidencing that large-scale amplification of the exogenous DNA had occurred. Similar results are depicted in DECLARATION 8.

Further, as described and exemplified in the specification, another way to identify amplified chromosomes, including sausage chromosomes and SATACs, is by *in situ* hybridization, including fluorescence *in situ* hybridization, for chromosomal structures co-stained for amplified heterochromatin and the heterologous DNA (*e.g.* containing the selectable marker, which by virtue of practice of the method integrates into an amplifiable region is amplified). The specification describes that sequences contained in the pericentric heterochromatin, which by practice of the method are amplified, include rDNA and/or satellite DNA (see *e.g.* at page 33, line 28 to page 34, line 2). In particular, the specification and Examples (see e.g. Example 6) describe identification of sausage chromosomes and SATACs by *in situ* hybridization for amplified heterochromatin using a probe for heterochromatin (e.g. a mouse satellite DNA probe) for identification of the amplified pericentric heterochromatin and/or heterologous DNA, (thus even the nucleic acid encoding the selectable marker can be used as a probe) (see *e.g.* at page 94, lines 10-16; at page 95, lines 5-9). Further, the Example shows that *in situ* hybridization for the amplified heterochromatin and the heterologous DNA co-localized (see *e.g.* at page 95, lines 5-9).

The DECLARATION 5 and 7 (Dr. Fabijanski) and DECLARATION 8 (Dr. Lindenbaum) identify chromosomes that have undergone amplification, including SATACs and sausage chromosomes, exactly as described in the specification, *i.e.* using *in situ* hybridization for co-localization of amplified heterochromatin and heterologous DNA. As

taught in the specification, the DECLARATIONS use probes to rDNA to identify the amplified heterochromatin. *In situ* hybridization, including fluorescence *in situ* (FISH) techniques, was an available technique in plants (see *e.g.*, Leitch *et al.* (1991) *Genome*, 34:329-333; Fukui *et al.* (1994) *Theor. Appl. Genet.*, 87:893-899; Jiang *et al.* (1995) *Proc. Natl. Acad. Sci.* USA, 92:4487-4491; Murata and Motoyoshi (1995) *Chromosoma*, 104:39-43; Matsuyama *et al.* (1996) *Genome*, 39:941-945; Zhong *et al.* (1996) *Chr. Res.*, 4:24-28; and Schubert I and Wobus U (1985) *Chromosoma*, 92:143-148) at the time of filing the application and priority applications. Using such methods, Applicant has identified SATACs in three diverse plant species, *Brassica* and *Nicotina* (DECLARATIONS 5 and 7) and soybean (DECLARATION 8). Demonstration of the identification of SATACs in three diverse plant species using methods as taught in the specification evidences the applicability of the teachings of the specification within the full scope of the claims.

Thus, the specification teaches numerous ways to identify SATACs based on characteristics contained within a SATACs that do not require identifying the centromere. As noted in a previous response, the Examples demonstrate use of an anti-centromere antibody to show that the exemplified SATAC contains a centromere, but this is not required when producing SATACs. There is no requirement in the specification that the centromere must be identified by an anti-centromere antibody in order to identify a SATAC. In fact, as discussed above, the specification teaches many other diverse ways of identifying SATACs based on their unique characteristics. Further, one of skill in the art can infer the presence of a centromere from the stable and extragenomic existence of the chromosome. For example, since a SATACs contains a centromere derived from the eukaryotic cell in which the amplification event took place, it is stable and can replicate and segregate alongside the endogenous chromosome (see e.g. at page 16, line 22 to page 17, line 4; page 34, line 23). As described in the specification, stability can be assessed in the presence or absence of a selective agent (at page 17, lines 11-14). Applicant provided evidence in DECLARATIONS evidencing such stability and segregation.

3) The Examiner states that:

> ***A single generation of plants grown in the absence of a selection marker is not sufficient to demonstrate stable inheritance patterns for an autonomously replicating chromosome with a fully functioning centromere.***

Applicant respectfully disagrees with the above assertion by the Examiner. The Examiner has provided no evidence or reason why stable inheritance was not sufficiently

demonstrated. As discussed above, the specification states that a SATAC is a stable, extragenomic artificial chromosome, which necessarily requires the presence of a centromere. Further, as discussed above and in the previous response, the specification states that stability of a chromosome can be measured in the presence or absence of selective agents (see e.g. at page 17, lines 10-14). The DECLARATIONS of record evidence maintenance of SATACS in the presence of selective agents over thousands of mitotic divisions. The DECLARATIONS of record also evidence maintenance under non-selective conditions through meiosis through its transmission to a second generation plant. Thus, contrary to the Examiner's assertion, stability was shown not only in a single generation of plant, but also into a daughter plant by virtue of entering the germline and being sexually transmitted to progeny.

For example, the DECLARATIONS of record demonstrate the teachings of the specification in plants and that resulting plant SATACS are stable. The stability of the SATACs generated in plants is verified in DECLARATION 5 and 7 by data evidencing maintenance of SATACs in the ***presence*** of a selective agent, which was achieved over **thousands of mitotic divisions**. Upon division of cells, if the SATAC did not contain a functioning centromere it would have been lost over this many cell divisions. This was not the case. Further, the DECLARATION 5 and 7 also showed stability of the SATACs through meiosis by its transmission to a second generation plant. DECLARATION 7 demonstrates that the transmission follows mendelian frequency, again an occurrence dependent on the presence of a functional centromere.

DECLARATION 8 of Dr. Lindenbaum demonstrates that the SATAC also is stably maintained mitotically and meiotically in the ***absence*** of selection and found in the **second** generation of a plant derived from a plant cell containing a SATAC. The results in DECLARATION 8 demonstrate that following introduction of heterologous DNA into embryogenic soybean cultures, the embryogenic clusters were grown in the absence of selective conditions to generate mature embryos, which were then desiccated in darkness for 5-7 days and then germinated. Within a few days, the roots emerged followed by shoots. The germinated embryos were planted and grown. FISH analysis of root tips of the regenerated embryos **AND** of T1 seeds obtained from self crosses of regenerated embryos demonstrated the presence of SATACs. Thus, as set forth in the DECLARATION 8, Dr. Lindenbaum stated that "the plant SATAC has a fully functional centromere that permits it to undergo mitosis in somatic cells as well as meiotic replication enabling it to enter the germ

line and be sexually transmitted to the T1 progeny." Thus, as stated in the last response, The DECLARATION evidences that the SATAC is stably maintained mitotically and meiotically in the absence of selection and found in the **second generation** of a plant derived from a plant cell containing a SATAC.

Thus, it respectfully is submitted that the Examiner's assertion that the DECLARATIONS do not evidence stability of a fully functioning centromere based on a single generation of plants grown under non-selective conditions is a conclusory statement with no support as to why this does not evidence stability. The Examiner has provided no scientifically sound evidence why Applicant's assertions and the DECLARATIONS, signed under penalty of criminal perjury, are not true. The Examiner is reminded that MPEP 2144.03 states:

> The Examiner may take official notice of facts outside of the record which are capable of instant and unquestionable demonstration as being "well- known" in the art. *In re Ahlert*, 424 F.2d 1088, 1091, 165 USPQ 418, 420 (CCPA 1970). . . .

The facts of which the Examiner is taking notice are conclusory and are not capable of instant and unquestionable demonstration as being "well-known" in the art. MPEP 2144.03 continues:

> If justified, the examiner should not be obliged to spend time to produce documentary proof. If the knowledge is of such notorious character that official notice can be taken, it is sufficient so to state. *In re Malcolm*, 129 F.2d 529, 54 USPQ 235 (CCPA 1942). If the applicant traverses such an assertion the examiner should cite a reference in support of his or her position.

In this instance, Applicant requests that support for the Examiner's position be provided, since the conclusory statement by the Examiner is not unquestionable or well-known.

I. REJECTION OF CLAIMS 50-52, 73-79, 81, 84, 87-95, 97-99, 101, 104, 108, 111, 114-115, 117, 119-121 AND 128 UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 50-52, 73-79, 81, 84, 87-95, 97-99, 101, 104, 108, 111, 114-115, 117, 119-121 and 128 are rejected under 35 U.S.C. §112, first paragraph, because it is alleged that the specification does not provide guidance for any plant artificial chromosome, sequences, or methods of making the same, or any plant cell comprising any artificial chromosomes.

Applicant respectfully traverses this rejection for reasons of record. Specifically, Applicant rebuts each of the Examiner's specific comments below.

Rebuttal to Examiner's comments:

4) The Examiner states that:

> ***It is not clear from the specification which methods may be used with plants and which may not. The listed identifications, G-banding, C-banding, immunoflurescence, electron microscopy and Southern***

blot all are methods that would NOT work in plants because all rely on features that are not present in plant cells and plant chromosomes.

Applicant respectfully disagrees with the Examiner's assertion. As discussed in previous responses, the cytological methods were well known to one of skill in the art at the time of filing the Application. Techniques such as chromosome banding, immunofluroescence, electron microscopy, southern blot, and *in situ* hybridization were known to one of skill in the art and had also been demonstrated as applicable with plant chromosomes as well as with plant cells (see for example, Plant Chromosomes: Laboratory Methods (ed. Kiichi Fukui and Shigeki Nakayama, 1996), available at http://books.google.com/books?id=cTRgMDnrMOoC&pg=PA46&lpg=PA46&dq=plant+chromosomes:+laboratory+methods&source=bl&ots=ilpBfCl2aa&sig=h4D6PEVZgX2fTg7A3R8QdFGmLiM&hl=en&ei=cINBSuOIHMfKlAfFw6DsCA&sa=X&oi=book_result&ct=result&resnum=1; Arun Kuman Sharma & Archana Sharma, Chromosome Techniques – A Manual (Harwood Academic Publishers, 1994), available at http://books.google.com/books?id=iQ0GWC_jBZwC&dq=Chromosome+Techniques+%E2%80%93+A+Manual&printsec=frontcover&source=bl&ots=VeI55J0U5Q&sig=Sa7z8Se1_-EEUnjB5UUMO3Wpq1I&hl=en&ei=noNBSuerG9GrlAfOrbD0CA&sa=X&oi=book_result&ct=result&resnum=1; Methods in Plant Cell Biology (ed. Galbraith *et al.* 1995), available at http://books.google.com/books?id=aGQ_gi7sOc8C&printsec=frontcover&source=gbs_v2_summary_r&cad=0; Sparvoli *et al.* (1994) J. Cell Science 107:3097-3103 and Wanner *et al.* (1995) Chromosome Res. 3:368-74). The books are available in part on line and the URL is provided. Applicant is willing to purchase copies of these books to provide to the Examiner if the Examiner requests copies.

In addition, as discussed in at least the previous two responses and in Declaration 7 of Dr. Fabijanski, fluorescence *in situ* (FISH) techniques were available at the time of filing the earliest priority application (see *e.g.*, Leitch *et al.* (1991) *Genome*, 34:329-333; Fukui *et al.* (1994) *Theor. Appl. Genet.*, 87:893-899; Jiang *et al.* (1993) 36:792-5; Jiang *et al.* (1995) *Proc. Natl. Acad. Sci.* USA, 92:4487-4491; Murata and Motoyoshi (1995) *Chromosoma*, 104:39-43; Matsuyama *et al.* (1996) *Genome*, 39:941-945; Zhong *et al.* (1996) *Chr. Res.*, 4:24-28; and Schubert I and Wobus U (1985) *Chromosoma*, 92:143-148).

Based on these references, it is unclear why the Examiner concludes that such techniques do NOT work in plants. In the previous Office Action, Applicant reminded the Examiner that MPEP 2144.03 states:

> The Examiner may take official notice of facts outside of the record which are capable of instant and unquestionable demonstration as being "well- known" in the art. *In re Ahlert*, 424 F.2d 1088, 1091, 165 USPQ 418, 420 (CCPA 1970). . . .

The facts of which the Examiner is taking notice are conclusory and are not capable of instant and unquestionable demonstration as being "well-known" in the art. MPEP 2144.03 continues:

> If justified, the examiner should not be obliged to spend time to produce documentary proof. If the knowledge is of such notorious character that official notice can be taken, it is sufficient so to state. *In re Malcolm*, 129 F.2d 529, 54 USPQ 235 (CCPA 1942). If the applicant traverses such an assertion the examiner should cite a reference in support of his or her position.

Applicant also stated and requested that if the rejection is maintained that *in situ* hybridization, including FISH, was not routine at the time of filing, documentation supporting this position, respectfully is requested. No documentation has been provided by the Examiner. Therefore, Applicant further requests that the Examiner provide support for the position that not only *in situ* hybridization but also the other stated methods such as southern blot would not work in plants. If such documentation cannot be provided, this rejection cannot be maintained.

Further, it respectfully is submitted that cytological techniques are not crucial to the claimed subject matter. Identification of cells by cytology is not a limitation stated in the claims. Further, other methods besides cytology permit the identification and isolation of SATACs. These include, for example, fluorescence-activated cell sorting, methods that distinguish the SATACs by size differences from endogenous chromosomes (e.g. swinging bucket centrifugation) and immunoaffinity procedures (see e.g. at page 41, line 5 to page 42, line 3). In addition, a SATAC can be introduced into a cell by microcell fusion without having to identify or isolate the SATAC (e.g. claim 74).

5) The Examiner states that:

> ***Applicants urge that fluorescence in situ hybridization was known in the art at the time of filing. This is not persuasive because Applicants are urging that the exact same steps and methods are being followed to generate plant SATACs. Different probes and different hybridization materials would have been required for plant in situ hybridization.***

The Examiner is reminded that the law of enablement does not require the Applicant to provide an example of every species or reagent required to practice the method. For example, "it is manifestly impracticable for an applicant who discloses a generic invention to given an example of every species falling within it, or even to name every such species, it is sufficient if the disclosure teaches those skill in the art what the invention is and how to

practice it." *In re Grimme, Keil and Schmitz*, 124 USPQ 449, 502 (CCPA 1960). Further, a finding of enablement is not precluded even if some experimentation is necessary, as long as the amount of experimentation is not undue. *Atlas Powder Co. v. E.I. DuPont De Nemours Co.*, 750 224 USPQ 409, 3 (Fed. Cir. 1984); *W.L. Gore and Associates v. Inc.*, 7212 220 USPQ 303, 315 (Fed. Cir. 1983). The scope of enablement is based on that which is disclosed in the specification plus the scope of what would be known to one of skill in the art without undue experimentation. *National Recovery Technologies, Inc. v. Magnetic Separation Systems, Inc.*, 166 F. 3d 1190, 49 USPQ 2d 1671 (Fed. Cir. 1999). A patent need not teach, and preferably omits, what is well known in the art. *In re Buschner*, 929 F.ed 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Linemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 739 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

As discussed above and further below, *in situ* hybridization, including FISH, was a well known technique in plants at the time the application was filed. There is no requirement that the Applicant provide every single possible probe that can be used to identify the SATAC, since such probes are dependent on the particular heterologous DNA that is introduced. The identification of probes to use to perform the *in situ* hybridization in plants is within the level of skill of one of skill in the art and does not require undue experimentation. Furthermore, as noted above, probes based on the sequence of the introduced DNA fragment, such as probes based on the sequence that encodes the selectable marker, can be used.

The specification teaches application of FISH for the identification of SATACs in mammalian cells for the presence of pericentric heterochromatin interspersed with heterologous DNA. The specification teaches that sequences contained in the pericentric heterochromatin, which by practice of the method are amplified, include rDNA and/or satellite DNA (see e.g. at page 29, lines 11-28). Example 6 in the specification teaches using *in situ* hybridization using probes to the amplified heterochromatin (i.e. a probe to mouse major satellite DNA) to identify SATACs by permitting identification of the amplified heterochromatin (see e.g. at page 94, lines 10-16). The Example also shows that *in situ* hybridization for the amplified heterochromatin and the heterologous DNA co-localized evidencing the presence of foreign DNA interspersed with the amplified heterochromatin (see *e.g.* at page 94, lines 10-16; at page 95, lines 5-9). A probe to hygromycin is an exemplary

probe to "foreign" DNA taught in the application (see e.g. at page 96, lines 19-29). The DECLARATIONS of record identify SATACs exactly as described in the specification using *in situ* hybridization for co-localization of amplified heterochromatin (probes to various rDNA, e.g. 18s rDNA probe in Brassica; 45S nuclear rDNA gene locus in Soybean) and heterologous DNA (probes to the selectable marker including to hygromycin).

Hence, the probes used in the DECLARATIONS were based on those **exactly** as taught in the specification, i.e. probes to the amplified pericentric heterochromatin (e.g. satellite DNA or rDNA) and to the heterologous DNA. The exact probes used are well within the level of skill in the art, for example, based on the particular heterologous DNA that is introduced into the cell to initiate the amplification events and the particular plant endogenous sequences of pericentric heterochromatin (e.g. rDNA) to identify SATACs containing amplified heterochromatin. For example, rDNA sequences in plants were known at the time of filing the specification, and were used to generate probes for *in situ* hybridization. For example, a review in 1994 by Jiang and Gill provided a summary of *in situ* hybridization techniques and their application in plant genome mapping, including the use of rDNA probes, See also, e.g. Maluszynska *et al.* (1991) Plant J., 1: 159-166; Maluszynska *et al.* (1993), 36:774-81; Bauwens *et al.* (1991) Chromosoma, 101-41-8; Griffor *et al.* (1991) Plant Mol. Biool., 17:101-9; Xu *et al.* (1994) Genome, 37:1062-5; Kamisugi *et al.* (1994) Mol. Gen. Genet., 245:133-8; Fukui *et al.* (1994) Genome, 37:105-11; Fuchs *et al.* 1994; Busch *et al.* 1995; Cuadrado *et al.* (1995) Genome, 38:795-802; Fominaya *et al.* (1995) Genome, 38:548-57; Moscone EA *et al.* (1996) Chromosoma, 105: 231-6; Schmidt *et al.* (1996) Plant Mol. Biol., 30: 1099-113; Shi *et al.* (1996) J Hered., 87:308-13; Hanson *et al.* (1996) Chromosoma, 105:55-61; Xu *et al.* (1996) Chromosoma, 104: 545-50).

Thus, it respectfully is submitted (as established in previous responses) that undue experimentation was not required to generate probes in the DECLARATIONs of record, e.g. probes to the 18s rDNA in Brassica (see e.g. Declarations 5 and 7), a probe to the 45S rDNA loci in soybean (see e.g. Declaration 8), or a probe to the introduced hygromycin foreign DNA (see e.g. Declarations 5 and 7). Also, generating probes to foreign DNA is not specific to any plant sequence. For example, just as taught in the specification, the DECLARATION 5 and 7 of Fabijanski utilized a probe to hygromycin. As stated in DECLARATIONS of Dr. Fabijanski, *in situ* hybridization is a standard method. Similarly, the generation and use of probes for *in situ* hybridization also is a standard method at the time the application was filed.

Accordingly, it respectfully is submitted that undue experimentation is not required to generate the specific probes used in the DECLARATIONs to identify SATACs. Further, as discussed above, FISH analysis is but one technique that can be used to identify SATACs. This technique in plants was demonstrated in the DECLARATIONS of record, which generated and identified SATACs based on the teachings of the specification and what was known to one of skill in the art at the time the application was filed.

6) The Examiner states that:

> ***Applicants urges primarily, that once the demonstration of SATAC formation of mammalian cells was disclosed, that it is not necessary to disclose the same detailed analysis to show the generation of SATACs in plants because the identifying characteristics are the same across species... This is not persuasive because the state of the art in mammalian cytology and artificial chromosome generation versus the state of the art in plant cytology and artificial chromosome generation are vastly different, even in the present day, more than a decade after filing and certainly at the time of filing...[T]he tools to perform the detailed analysis for plants were not known at the time of filing. It is submitted therefore, that it would not have been possible to know that the identifying characteristics of SATACs were the same between mammalian SATACs and plant SATACs because it wasn't possible to identify the same structural features in plants that were identified and demonstrated in the specification.***

SATACs in plants have the same identifying features as SATACs produced in any other eukaryotic cells, including in mammalian cells. This is demonstrated in the DECLARATIONs of record. The specification states that plant satellite artificial chromosomes have the same structural elements as described for mammalian artificial chromosomes, except that they have a plant centromere (see for example, at page 16, lines 27-29), which centromere is produced by virtue of production of the SATAC in a plant. This is so by virtue of the universality of the method, which is based on processes that are fundamental to chromosomal replication in all cells. The specification sets forth these identifying characteristics as discussed in detail in this and previous responses, and the DECLARATIONS of record demonstrate the generation of SATACs based on such identifying characteristics. Since the specification sets forth the identifying characteristics of SATACs, generated in any eukaryotic species, the Examiner's statement above is inapt.

The specification unequivocally establishes that the same method can be used in plants to generate SATACs containing the same relevant identifying characteristic of a SATAC applicable to all species. As described in the specification, this method is

generalizable and universal to all chromosomes and species (see *e.g.* at page 36, lines 3-4), thereby generating species-specific chromosomes. The application specifically teaches that the process can be applied to any species, including plants and animals. For example, the specification teaches generically the steps of the method (*e.g.* at page 6, lines 15-24):

> The chromosomes provided herein are generated by introducing heterologous DNA that includes DNA encoding one or more multiple selectable marker(s) into cells, preferably a stable cell line, growing the cells under selective conditions, and identifying from among the resulting clones those that include chromosomes with more than one centromere and/or fragment thereof. The amplification that produces the additional centromere or centromeres occurs in cells that contain chromosomes in which the heterologous DNA has integrated near the centromere in the pericentric region of the chromosome. The selected clonal cells are then used to generate artificial chromosomes.

And further states at page 9, lines 14-18:

> These methods are applicable to the production of artificial chromosomes containing centromeres derived from any higher eukaryotic cell, including mammals, birds, fowl, fish, insects and plants.

Thus, the recognition of the universality of the method was evident at the time of filing the instant application. As discussed in previous responses, DECLARATION 6 of Dr. Hadlaczky states as a fact that the method is universal based on fundamental chromosomal processes in animals and plants. Further, it is clear from the specification that the only difference between a plant SATAC and a mammalian SATAC is that the former contains a plant centromere, which is necessarily because practice of the method in a plant cell results in de novo formation of the centromere from the endogenous centromere. As discussed elsewhere herein and in previous responses, it is not necessary to identify the centromere to identify a SATAC because of the presence of the other unique identifying features of all SATACs, such as the presence of more heterochromatin then euchromatin interspersed with heterologous DNA on a stable extragenomic chromosome. The DECLARATIONs of record demonstrate that SATACs generated in plants have these same identifying characteristics exactly as described in the specification. Thus, the DECLARATIONS prove the reproducible generation of SATACs in plants exactly as taught in the specification. Thus, the Examiner's assertions are not sustainable. The fact that persons of skill in the art, based on the teaching of the specification, can generate and identify SATACs in plants has been established.

7) The Examiner states that:

> ***Applicant urges that the specification does not require the***

absence of a selective agent for the measurement of stability of the chromosome and that DECLARATION 3 provides evidence that such stability was exhibited even in the absence of a selective agent (see pages 16-18 of response).

This is not persuasive because as discussed above and in the other office actions, there was additional evidence available for the presence of a centromere and centromeric sequences in mammalian SATACs. As discussed previously, in the absence of such evidence and in the absence of similar tools, a different standard must be applied to plants to show the presence of a centromere as required by SATACs according to the definition of the instant specification.

Applicant has rebutted this assertion above under headings 2) & 3) above. To repeat, as stated extensively in this and previous responses, no knowledge of the centromere is required to generate or identify SATACs. That is a benefit of the instant method. **The specification does not state that identification of the centromere is required to identify a SATAC.** In contrast, the specification provides detailed description of relevant identifying characteristics that can be used to identify a SATAC in any species, and uses such characteristics to identify SATACs in mammals (see e.g. Figure 2 and 3 and Example 6). These include, for example, the use of southern blot to evidence amplification of the heterologous DNA, *in situ* hybridization for the identification of chromosomal structures, including extragenomic chromosomal structures, that have more heterochromatin then euchromatin and that are interspersed with the foreign DNA, and evidence of stability of the SATAC in the presence or absence of a selective agent. The use of an antibody to the centromere in the specification was merely used to aid in the initial detailed characterization of the SATAC. Once characterized, the use of an antibody to the centromere is not required. As discussed above, the exact methods used to identify SATACs in the specification were demonstrated in the DECLARATIONS for generation and identification of SATACs in plants.

8) The Examiner states that:

Furthermore the transmission does not even definitively show the presence of a centromere. For example, in a study of the transmission of acentric chromosomes Kanda et al (2000 J. Cell Biochem Suppl. 35:107-114) disclose that some acentric chromosomes are stably transmitted through successive cell divisions. Kanda et al propose that chromosomes may "tether" to a host chromosome and show stable transmission (see abstract and page 109 in particular). The acentric chromosomes in this case use the cells machinery to replicate and are not

autonomously replicating (see page 109). Given the state of the art, the mere presence of a chromosomal fragment does not give evidence of a SATAC as defined by the specification.

The above statement by the Examiner is based purely on speculation and conjecture. The Examiner has provided no sound proof that the SATACs exemplified in the DECLARATIONS of record are tethered to a host chromosome. In fact, there is no tethering evident in the figures in the Declarations. For example, DECLARATION 7 of Dr. Fabijanski established that the resulting SATACs, transmitted through meiosis, exhibit mendelian characteristics. It is unclear based on the Examiner's theory, how a chromosomal structure that is tethered to a host cell chromosome could exhibit a segregation pattern at a particular Mendelian frequency. Further, the DECLARATION 8 of Dr. Lindenbaum states that "[a]nalysis of hybridized spreads at higher magnification...show that it is clear that the SATAC is, in fact, separate and autonomous of other cellular chromosomes." In view of the statements of the signed Declarants, and without additional evidence that contravenes the data provided to the Examiner, the Examiner's statement suggesting that evidence of SATAC stability is because they are tethered to a host cell chromosome is inappropriate and fanciful.

9) The Examiner states that:

Applicants urge that cited references do not take into account the disclosure of the instant specification in regard to SATACs (see page 19-20 of response).

This is not persuasive because the state of the art has been established. The teachings of the specification are taken into account upon examination and are found to not give guidance in enough detail, particularly because this is a pioneering technology, for one of skill in the art to make and use and practice the instantly claimed invention.

Applicant has rebutted this assertion under heading 1) above.

10) The Examiner states that:

Applicants attention is drawn to claims that are drawn to isolating the SATAC and directly introducing the SATAC into plant cells. There is no support literally, or in DECLARATIONS to indicate that this may be done with plant SATACs, particularly at the time of filing when chromosomal manipulation in plants was in its infancy. It is precisely because of the novelty aspect of the invention that a detailed disclosure is required for enablement. One of skill in the art would have appreciated at the time of filing the vast differences between plant and mammalian cells and chromosomes and would not have expected routine experimentation to result in the successful generation of SATACs in plants. The specification does not show or disclose the

successful generation of SATACs in plants.

Although the comment by the Examiner is set forth under an enablement rejection, the specific comment implies that the Examiner believes that there is not sufficient support in the specification for generating plant SATACs or introducing SATACs into plant cells. Although the rejection for lack of written description is addressed below, Applicant rebuts the Examiner's comments here too.

Applicant respectfully disagrees for reasons of record in previous responses. First, the specification teaches a universal method of generating SATAC in any species based on a process fundamental to chromosomal replication in all cells. Thus, any differences in knowledge of chromosomal structure and manipulation is irrelevant to the method. The method, and the resultant SATACs, require no chromosomal manipulation.

In addition, the Declarations unequivocally demonstrates and the specification unequivocally states that the method is universal and can be applied to plants and animals. For example, and as discussed further below, the specification states at page 9, lines 15-18 that:

> These methods are applicable to the production of artificial chromosomes containing centromeres derived from any higher eukaryotic cell, including mammals, birds, fowl, fish, insects and **plants**.

And further at page 30, lines 9-12 states that:

> The following discussion describes this process with reference to the EC3/7 line and the resulting cells. The same procedures **can be applied to any other cells**, particularly cell lines to create SATACs and euchromatic minichromosomes.

Based on the above, and the fact that the **specification includes an entire section devoted to introduction of DNA into plant cells** (see.*e.g.* at page 54, line 1 to page 55, line 3 under the section headed "Introduction of heterologous DNA into plants"), which is the procedure that initiates the amplification events, it is **unequivocally clear** that the specification describes and teaches a universal process that works equally in plants and animals. The steps of the methods generically described in the application, and exemplified using EC3/7 cells, for the generation of artificial chromosomes, such as SATACs and sausage chromosomes, works exactly the same in plants.

In addition, besides teaching in detail the method of introducing DNA into plant cells to initiate the amplification process that result in the generation of SATACs and intermediates and precursors thereof, the specification also includes an entire section on the

introduction of SATACs (any SATAC, plant or animal) into plant cells. For example, the specification describes that if the artificial chromosome is contained within a cell, it can be introduced by cell fusion or microcell fusion. Other techniques include, but are not limited to, direct DNA transfer, electroporation, lipid-mediated transfer, microprojectile bombardment, microinjection or PEG-induced DNA uptake, see, for examples, at page 48, line 11 to page 56, line 21, which includes description of introducing artificial chromosomes into plants or heterologous DNA into plants to generate SATAC using the methods described in the application.

There is no requirement for providing Working Examples. "It is not necessary that a patent applicant test all the embodiments of an invention." Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd., 18 USPQ2d 1016 (Fed. Cir. 1991); In re Angstadt, 190 USPQ 214, 218 (CCPA 1976). " Representative samples are not required by '112, "and are not an end in themselves." In re Robins, 166 USPQ 552, 555 (CCPA 1970). In re Anderson, 471 F.2d 1237, 176 USPQ 331 (CCPA1973):

> The specification is presumed accurate. Absent reasons for doubting the objective truth of statements in the specification, applicant has complied with the enablement requirement of 35 U.S.C. §112, first paragraph.

In re Marzocchi & Horton, 58 CCPA 1069, 439 F. 2d 220, 169 USPQ 367, 369-370 (1971):

> [a]s a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and describing the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of ' 112 unless there is reason to doubt the objective taught of the statements contained therein which must be relied on for enabling support.

Notwithstanding this, the Working Examples in the application exemplify practice of the method generally, and show how to perform the methods and characterize the resulting chromosomal structures. Contrary to the assertions by the Examiner, the Working Examples were provided to show and confirm practice of the method in an exemplary cell, which is equally applicable to other cells based on the fundamental processes that occur upon introduction and incorporation of heterologous DNA into an amplifying region of a chromosome of any cell, plant or animal. The DECLARATIONS of records confirm practice of the method as taught in the specification in plants.

11) The Examiner states that:

> ***No publications of record in the decade since the time of filing have shown the successful generation of SATACs in plants.***

The fact that no publication of record has shown the successful generation of SATACs in plants is not dispositive of a lack of enablement. Furthermore, as stated to the Examiner in previous Office Actions, Agrisoma, the licensee of the application at issue, is a fully funded company that was formed to commercialize the plant satellite artificial chromosomes. Agrisoma has generated and commercialized plant satellite artifical chromosomes. As a commercial, not institutional entity, there is no reason to publish. As described in the DECLARATION 7 of Fabijanski, Agrisoma produces plant satellite artifical chromosomes and other intermediates, such as cells with sausage chromosomes, as described in the above-captioned application as well as parent applications. As stated Agrisoma did not further develop the technology, but has used the technology as described in the application. Clearly, this entity exists and its products are fungible. There can be no better proof of the successful generation of such structures, then the formation of a company based upon such structures.

12) The Examiner states that:

> ***The DECLARATIONs of record show chromosomal fragments in cells with no demonstration that such fragments, are in fact, SATACs. For these reasons, in addition to the reasons of record, it is incumbent on Applicants to provide a full disclosure of the technology to be patented.***

The Examiner continues to question the validity of assertions made by the undersigned and in the DECLARATIONS under penalty of perjury that evidence generation of SATACs in plants. The Examiner has provided no sound reasoning for the assertion that the DECLARATIONS only evidence "chromosomal fragments" and do not demonstrate SATACs. To the contrary, Dr. Fabijanski and Dr. Lindenbaum, persons of skill in the art in the field, have attested otherwise. The undersigned reminded the Examiner in the Response mailed January 31, 2008, that there is a presumption that statements made in applications are true, and that DECLARATIONS include a penalty clause. There is guidance on this matter at MPEP 2107, and case law cited below:

> It is incumbent upon examiner to first establish a prima facie case of non-enablement (In re Arbruster, 512 F.2d 76, 185 USPQ 152 (CCPA 1975); In re Marzocchi, 439 F.2d 220, 169 USPQ 367 (CCPA 1971)[a]s a matter of Patent Office practice, tehn, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of §112

> unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.
>
> ...it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure (In re Marzocchi & Horton, 58 CCPA 1069, 439 F.2d 220, 169 USP 367, 369-370 (1971)).

See also, e.g. MPEP 2107:

> Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill int eh art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

The Examiner has not provided any objective reason to doubt the truth of the statements made by Dr. Fabijanski and Dr. Lindenbaum who have attested in DECLARATIONS the generation of SATACs in plants.

12) The Examiner states that:

> ***Applicants urge that the specification is not limiting to the ways of generating SATACs and that size and number of chromosomes do not need to be known to generate SATACs and that Example 10 is not the only way to isolate SATACs (pages 21-22 of response).***
>
> ***These are not persuasive arguments because one of skill in the art is not given sufficient guidance to know which methods would work and which would not work in plants. Furthermore, as discussed above, wherein the tools and information available for mammalian cells are not available for plant cells, additionally information is necessary to determine success. The specification does not give guidance for determining this information or give guidance indicating the necessity of this information. See Genentech, Inc. v. Novo Nordisk, A/S, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997), which teaches that disclosure of a "mere germ of an idea does not constitute [an] enabling disclosure", and that "the specification, not the knowledge of one skilled in the art" must supply the enabling aspects of the invention.***

Applicant's arguments in the previous Office Action stated that the description at page 126-128 relating to particular cells lines and culturing procedures refers to the choice of cell in order to permit *large-scale production* of artificial chromosomes. The instant claims

are directed to methods of generating a transgenic plant and do not require large-scale isolation of SATACs. Hence, the teachings of the specification at pages 126-128 are irrelevant to the instant claims.

Further, Applicant stated that the method of isolating SATACs described in Example 10 is not the only way to isolate SATACs. Exemplary methods of isolating SATACs are described in detail in the specification beginning at page 41 under the heading "Isolation of Artificial Chromosomes." Notwithstanding this, the specification describes that SATACs can be isolated by fluorescence-activated cell sorting (FACs) by taking advantage of the nucleotide base content of the SATACs, by virtue of their high heterochromatic DNA content, which differs from any other chromosome in a cell (see *e.g.* at page 41, lines 7-11). Isolation of chromosomes in plants by FACs was well known to one of skill in the art at the time of filing the instant application and priority applications (see *e.g.*, de Laat and Blaas (1984) Theor Appl. Genet., 67:463-467). Furthermore, it was well known at the time of filing the instant application that heterochromatin, including plant heterochromatin, has a distinct base content (Guerra (2000) Genetics and Molecular Biology, 23:1029-1041 and references cited therein; Martin *et al.* (1988) Heredity, 61:459-467).

The disclosure provided in the specification for methods of generating SATACs, identifying SATACs and isolating SATACs is generic and universal to any SATAC generated from any eukaryotic cell, plant or animal. Thus, the description above for isolating SATACs, applies equally to plant or animal SATACs. It would not require undue experimentation for one of skill in the art to isolate SATACs using such techniques, since such techniques were well known at the time of filing the application. The Examiner has provided no reasoning or scientific basis why any of these methods would not work in plants.

Fairness

Applicant is entitled to claims that are commensurate in scope not only with what applicant has specifically exemplified, but commensurate in scope with that which one of skill in the art could obtain by virtue of that which the applicant has disclosed. In the above-captioned application, Applicant discloses to the public pioneering methods and compositions for the controlled introduction and stable extra-genomic maintenance of large heterologous DNA fragments in cells, and the generation of transgenic plants therefrom. It is clear that Applicant's discovery is of a pioneering nature, and, as such, is entitled to broad claim protection. It would be unfair and contrary to the public policy and to the Constitutional mandate set forth in Article, Section 8, to require Applicant to limit the claims

to methods using mouse SATACs. To do so would permit those of skill in the art to practice what is disclosed in the application but avoid infringing so-limited claims. To so limit the claims is contrary to the public policy upon which the U.S. patent laws are based. See, for example, *In re Goffe*, 542 F.2d 801, 166 USPQ 85 (CCPA 1970):

> for the Board to limit appellant to claims involving the specific materials disclosed in the examples so that a competitor seeking to avoid infringing the claims can merely follow the disclosure and make routine substitutions "is contrary to the purpose for which the patent system exists - to promote progress in the useful arts."

The public purpose on which the patent law rests requires the granting of claims commensurate in scope with the disclosure. This requires as much the granting of broad claims on broad inventions as it does the granting of more specific claims on more specific inventions. *In re Sus and Schafer*, 49 CCPA 1301, 306 F.2d 494, 134 USPQ 301, at 304.

II. The Rejection of Claims 1-5, 10-18, 20-22 and 24-27 Under 35 U.S.C. §112, First Paragraph – Written Description

Claims 1-5, 10-18, 20-22 and 24-27 and 28 are rejected under 35 U.S.C. §112, first paragraph for alleged lack of written description, because it is alleged that the specification does not describe the subject matter in such a way as to convey to one skilled in the relevant art that the inventor(s) had possession of the claimed subject matter at the time the application was filed. The undersigned assumes that the Examiner meant to reject the pending claims, which are 50-52, 73-79, 81, 84, 87-95, 97-99, 101, 104, 108, 111, 114-115, 117, 119-121 and 128. The claims are rejected for reasons of record that the specification allegedly only provides guidance for a satellite artificial chromosome in mammalian cells, specifically mouse cells, and does not provide guidance for any plant artificial chromosomes, sequences, or methods of making same, or any plant cells comprising any artificial chromosomes.

Applicant respectfully traverses this rejection for reasons of record. Specifically, Applicant rebuts each of the Examiner's specific comments below.

Rebuttal to Examiner's Comments:

13) The Examiner states:

Applicants urge that the specification clearly describes the relevant identifying characteristics of SATACs (see pages 25-26 of response).

This is not persuasive because as previously discussed, many of the characteristics regarding identification (i.e. g-banding and c-banding, and centromeric regions) for mammalian SATACs can not readily be applied to plant SATACs.

It respectfully is submitted that this rejection has been addressed in rebuttal 4) above in addressing the enablement rejection. First, it respectfully is submitted that such techniques can be applied to plants, and the Examiner has shown no evidence otherwise.

Second, the specification provides exhaustive detail of the structure of SATACs, which apply equally to plants or animals. The only difference between a plant SATAC compared to a mammalian SATAC is that the former contains a plant centromere. The application makes clear that the common attributes possessed by the members of the genus of satellite artificial chromosomes are relatively invariant: they have more heterochromatin than euchromatin and generally contain duplicated segments of DNA, which includes highly repetitive DNA, such as, for example, pericentric heterochromatic DNA or satellite DNA (see *e.g.*, page 7, lines 15-20). Furthermore, all arise by the amplification events as described in the application. The application describes that, except for the heterologous nucleic acid such as a selectable marker or other foreign DNA, the SATACs contain predominantly non-protein encoding heterochromatin (see page 7, lines 17-22). The application describes that this megachromosome (i.e. SATAC) is stable and can replicate and segregate alongside an endogenous chromosome (see e.g., page 16, lines 22-35; page 34, line 23).

Based on these identifying characteristics, the specification describes how to identify a satellite artificial chromosome, for example, by C-banding and/or fluorescence *in situ* hybridization (FISH) using labeled probes to visualize the amplified heterochromatin and the heterologous DNA. Finally, the DECLARATIONS of record evidence identification of SATACs in plants based on these identifying characteristics common to all SATACs using the exact techniques disclosed in the application and known to one of skill in the art at the time the application was filed. Thus, Applicant clearly described the identifying features of all SATACs, as they apply to plants and animals.

14) **The Examiner states:**

> ***Applicants urge that there is no requirement that all species within a genus be reduced to practice, nor that the specification include all examples of all species within a genus (page 26 of response).***
>
> ***This is not persuasive because the genus referred to by Applicant is already patented. However because this case is drawn to plant SATACs the genus of the instant case is the generation of plant SATACs. There are no embodiments described or disclosed and there are no working examples.***

As stated by the Applicant in previous Office Actions, there is no standard for patentability requiring a Working Example for everything claimed. It respectfully is submitted that Applicant has described in detail SATACs, including plant SATACs. To repeat from previous responses, as described in the specification, the method is generalizable and universal to all chromosomes and species (see *e.g.* at page 36, lines 3-4), thereby generating species-specific chromosomes. The application specifically teaches that the process can be applied to any species, including plants and animals. For example, the specification teaches generically the steps of the method (*e.g.* at page 6, lines 15-24):

> The chromosomes provided herein are generated by introducing heterologous DNA that includes DNA encoding one or more multiple selectable marker(s) into cells, preferably a stable cell line, growing the cells under selective conditions, and identifying from among the resulting clones those that include chromosomes with more than one centromere and/or fragment thereof. The amplification that produces the additional centromere or centromeres occurs in cells that contain chromosomes in which the heterologous DNA has integrated near the centromere in the pericentric region of the chromosome. The selected clonal cells are then used to generate artificial chromosomes.

And further states at page 9, lines 15-18:

> These methods are applicable to the production of artificial chromosomes containing centromeres derived from any higher eukaryotic cell, including mammals, birds, fowl, fish, insects and plants.

And further at page 30, lines 9-12 states that:

> The following discussion describes this process with reference to the EC3/7 line and the resulting cells. The same procedures **can be applied to any other cells**, particularly cell lines to create SATACs and euchromatic minichromosomes.

The description in the specification renders it clear that Applicant's were in possession of a method of generating amplified chromosomes in plants because there is an entire section in the specification devoted to exemplary plant hosts and methods of introducing heterologous DNA into plants (see.*e.g.* at page 58, line 1 to page 59, line 5 under the section headed "Introduction of heterologous DNA into plants"). The steps of the method are the same in all species.

Thus, although a Working Example was not specifically provided in plants, the specification renders it clear that the same procedures can be applied to any other cell to create SATACs. The inclusion of several pages devoted to a description of plant "hosts" and method to "introduce heterologous DNA into plants" to initiate the amplification events is a

sufficient description for one of skill in the art to practice the method as claimed. The specification provides detail of exemplary plants, methods of introducing DNA into plants, relevant identifying features of SATACs common to all eukaryotic species, mammals and plants, and methods to identify the resulting structure. Applicant steadfastly maintains that the based on this description one of skill in the art could practice the subject matter as claimed.

15) The Examiner states that:

> **Applicant requests the Examiner point out where in the specification the known centromeres for mammalian SATACs are found and urge that none of these features are necessary for the generation of SATACs (pages 27-29 of response).**
>
> **This is not persuasive because the specification on page 73, identifies sequences, mouse minor satellite DNA that specifically localizes to the centromeres of all mouse chromosomes. The specification also describes antibodies to known centromere associated proteins in mammalian cells for the recognition of SATACs, another tool not available for the recognition of plant SATACs.**

It respectfully is submitted that the Applicant requested the Examiner to point out where in the specification **SEQ ID NOS** for mammalian centromeres were provided in the specification, since in the last Office Action the Examiner stated that "while these structural features have been described and disclosed for mammalian centromeres and origins of replication in the form of SEQ IDs, these structures have not been disclosed for plant centromeres or origins of replication." Therefore, Applicant presumes that the Examiner could find no such support, since none exists. As stated by the Applicant throughout this response and previous responses, no knowledge of any centromere sequence is required to practice the method as claimed and no human centromeres were provided or used. Further, DNA fragment lambda CM8 does not contain a human centromere (see e.g. Cooper et al. (1992) Human Mol. Genetics, 1:753-754; and McGill et al. (1992) Human Mol. Genetics, 1:749-751). λCM8 is merely and exemplary DNA fragment used in the Examples in mammalian cells that can be introduced into the cell to initiate amplification events.

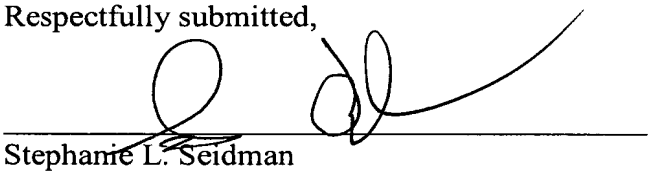
As discussed in the previous response, the use of anti-centromere antibodies or mouse minor satellite DNA probes, is only one way to show that the SATAC contains a functioning centromere. Assessment of stability and maintenance of the SATAC under selective and non-selective conditions is another way based on analysis of identifying characteristics. For example, Example 6 depicts identification of a SATAC using a probe to mouse major

satellite DNA, which recognizes the constitutive heterochromatin and a probe to heterologous DNA, which co-localized with the mouse major satellite DNA segments by *in situ* hybridization. This is one way to identify a SATAC. Staining for the centromere is not necessary, since presence a centromere can be evidenced and inferred by the fact that the SATACs function as a stable chromosome.

* * *

In view of the amendments and remarks herein, examination on the merits respectfully is requested.

Respectfully submitted,



Stephanie L. Seidman
Reg. No. 33,779

Attorney Docket No.: 0119354-00002/402E
Address all correspondence to 77202
Stephanie Seidman
K & L Gates LLP
3580 Carmel Mountain Road, Suite 200
San Diego, California 92130
Telephone: (858) 509-7410
Facsimile: (858) 509-7460
Email: stephanie.seidman@klgates.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Hadlaczky *et al.*	Art Unit	: 1638
Serial No.	: 09/724,726	Examiner	: Brent T. Page
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Title : ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

ATTACHMENTS

Chromosoma (Berl) (1985) 92:143–148

CHROMOSOMA

In situ hybridization confirms jumping nucleolus organizing regions in *Allium*

I. Schubert and U. Wobus

Zentralinstitut für Genetik und Kulturpflanzenforschung der AdW der DDR, DDR-4325 Gatersleben, German Democratic Republic

Abstract. In situ hybridization with a ^{125}I-rDNA clone from *Vicia faba* was performed against *Allium cepa* and three strains of top onion, which represent hybrids between *A. cepa* and *A. fistulosum*. In principle, the labelling patterns correspond to the patterns of the silver-stained nucleolus organizing regions (NORs) in the same species. This strongly supports the inference drawn from the Ag-NOR patterns that NORs can jump between terminal heterochromatin blocks of different *Allium* chromosomes in the parental species *A. cepa* as well as in their interspecific hybrids.

Introduction

Extensive genetic and molecular studies on the widespread occurrence of mobile genetic elements during recent years has led to far-reaching conclusions as to the functional and evolutionary significance of DNA transposition. However, the number of species and transposable sequences studied is still very limited in higher plants. One of us postulated earlier that nucleolus organizing regions (NORs) in some onion species behave like mobile genetic elements (Schubert 1984). This conclusion was based on the variability in size, number, and chromosomal position of active NORs and the number and size of interphase nucleoli after $AgNO_3$ staining in *Allium cepa* and *A. fistulosum* and their interspecific hybrids.

The following established phenomena (for review see Rieger et al. 1979) were considered as possible explanations for intraspecific variability between nonhomologous NORs: (1) differential amphiplasty (Navashin 1927, 1928) implicating repression in species hybrids of NOR(s) of one parental species; (2) nucleolar dominance, when as in barley (Nicoloff et al. 1979) two NORs are combined in one chromosome and one supresses the activity of the other; and (3) chromosomal interchanges involving regions containg NORs, translocating them into a new chromosomal position. Differential amphiplasty was excluded as an explanation since (a) variability occurred also in parental species and (b) the NORs of both parental species could be expressed in the hybrid species. Nucleolar dominance was also exlcuded because no combination of two NORs in one chromosome occurred. Finally, in a parallel investigation Giemsa banding patterns (Schubert et al. 1983) gave no evidence for gross chromosome interchanges in parental or in hybrid species.

However, no definite decision was possible as to whether differential activation or mobility of NORs was responsible for the variability observed, since silver staining labels only actively transcribing NORs. Therefore in situ hybridization was performed to localize active as well as inactive rDNA. The results strongly support the mobile NOR hypothesis. The NORs of *A. cepa* and possibly also those of *A. fistulosum* are, in all probability, able to move between telomeric heterochromatin blocks of different chromosomes.

Material and methods

Lateral roots of bulbs of *A. cepa* and different strains (15, 124, and 437) of top onions from the Gatersleben gene bank collection were used. The hybridogenic origin of the top onions [*Allium* × *proliferum* (Moench) Schrad.] from *A. fistulosum* L. and *A. cepa* L. was substantiated by Giemsa banding and $AgNO_3$ staining of NORs (Schubert et al. 1983).

Preparation of slides. Roots were treated with 0.05% colchicine for 2.5 h, then fixed in ethanol/acetic acid (3/1), digested in 1% pectinase (Serva) and 2% cellulase (Serva) for 2 h at 37° C in 0.1 M sodium citrate buffer, pH 4.7. Squashing was performed by the dry ice method.

Probe labelling. A plasmid (VER 17) with a 3.7 kilobase pair (kb) rDNA fragment from *Vicia faba*, cloned in pBR325 and containing parts of the 18S, the 5.8S and a large part of th 25 S rRNA coding regions as well as internal transcribed spacers (see Yakura and Tanifuji 1983), was labelled by nick translation with ^{125}I-dCTP (~68 TBq/mmol, Amersham) to a specific activity of $\sim 10^8$ dpm/µg.

Hybridization procedure. Freshly prepared and air-dried slides or slides stored in glycerol were washed twice for 5 min and once for 20 min in 2 × SSC (sodium chloride/sodium citrate) and once for 30 min at 60° C in 2 × SSC; then placed in 70% and 96% ethanol for 10 min each; and air dried. Then 20 µl RNase (100 µg/ml 2 × SSC) was applied to each slide for 40 min at 24 °C. Slides were washed twice for 5 min and once for 30 min in 2 × SSC; then subjected to 5 min postfixation in 4% paraformaldehyde in phosphate-buffered saline (PBS) and two 3-min washes in PBS; dehydrated through 30%, 60%, 80%, 96%, 100% ethanol for 10 min each; and air dried. A 10-µl sample

of the labelled probe, corresponding to about 130000 dpm in 3 × SSC + 0.1 M KI/50% deionized formamide, 10% dextranesulfate, was applied to each slide, and the coverslips were sealed with rubber cement. In situ denaturation took place for 2 min at 80 °C, and hybridization at 30 °C for 40 h, followed by two 5-min washes and five 30-min washes in 500 ml 2 × SSC + 0.1 M KI. After 10 min each in 70% and 96% ethanol both containing 0.3 M ammonium acetate and air drying, slides were dipped in ORWO K6 photoemulsion (containing 0.3 M ammonium acetate) and air dried again. After exposure at 4 °C in the dark, slides were developed and stained in 4% Giemsa (Merck) solution for 10 min and made permanent by euparal mounting.

Results

Our data demonstrate detection of rDNA sites in metaphase chromosomes of *Allium* species by in situ hybridization. Homologous hybridization (to *V. faba* root tip meristem slides) resulted in intensive labelling of nucleoli and NORs after 6–10 days (results not shown here). Exposure times of 4–5 weeks were necessary for heterologous hybridizations. This agrees with the observation of Knälmann and Burger (1979) that ^{3}H-rRNA of *V. faba* labelled interphase nucleoli of *A. cepa* with about one-third the efficiency observed for homologous hybridization.

Allium cepa

Five slides from roots of different individual bulbs of a strain without true heterochromatic satellites at the original nucleolar chromosome pair were evaluated. They showed two to four labelled sites on metaphase chromosomes (Table 1, Fig. 1 b, c). In two individuals a positive signal was found exclusively at the end of the short arm of the 'standard' nucleolar chromosomes, which normally possess a small heterochromatic satellite distal to the NOR.

Another individual most frequently showed four hybridization signals, two at the regular nucleolar chromosomes and two at the end of the short arm of the shortest submetacentric chromosome pair. Comparable results were obtained with $AgNO_3$ staining in *A. cepa* by Sato (1981). In a further individual, the main number of rDNA sites was three, two at the regular nucleolar chromosomes and one at the end of the short arm of one chromosome of the smallest pair. In the fifth slide, two to four signals were observed on metaphase chromosomes, the third and fourth signals (again in the smallest chromosomes) were, however, significantly weaker than those at the NOR sites of the usual nucleolar chromosomes.

Top onion strain 124

In this line, satellites of both parental nucleolar chromosomes were missing. Four slides from different individuals were evaluated. Between one and four, but most frequently two or three, metaphase chromosomes were labelled by ^{125}I-rDNA. The strongest labelling was on the NOR of the *A. cepa* nucleolar chromosome, the next strongest was either on a smaller submetacentric chromosome (probably the smallest one of *A. cepa*) or a somewhat larger nearly metacentric chromosome and a large submetacentric one of *A. cepa* (all labelled at the end of the short arm). The weakest signal was found at the NOR of the original *A. fistulosum* nucleolar chromosome (Table 1, Fig. 2a–c). The results confirm data obtained by $AgNO_3$ staining (Schubert 1984), except that the original nucleolar chromosome of *A. fistulosum* and not that of *A. cepa* showed the most

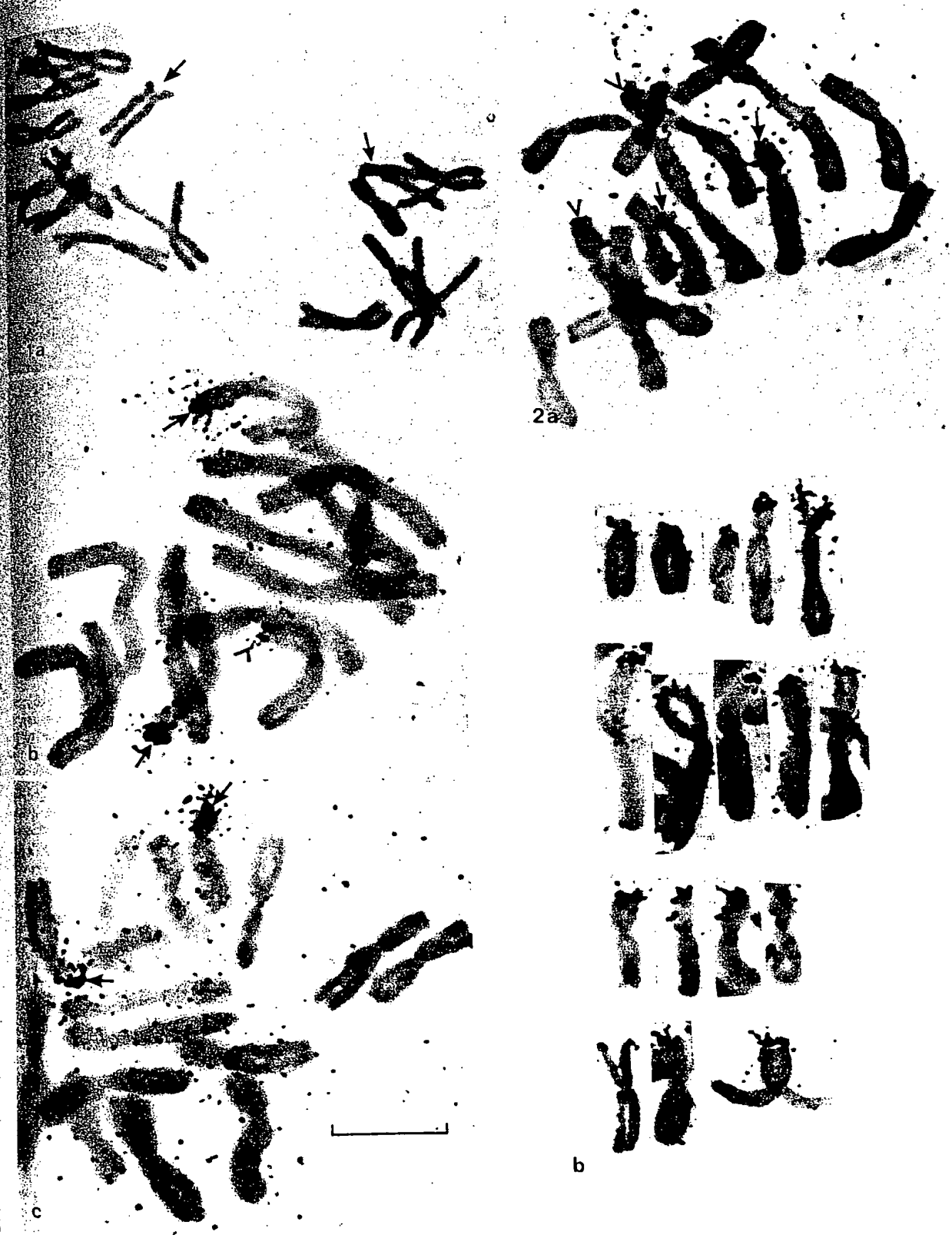
Table 1. Numbers of metaphases with 1, 2, 3, or 4 sites labelled by rDNA in individual slides of *Allium cepa* and top onion strains 15, 124 and 437[a]

	Allium cepa					Top onion strain 124					Top onion strain 437					Top onion strain 15		
Slide No.	3	8	13	18	23	10	15	20	24	Σ	11	16	21	25	Σ	9	14	19
No. of NORs																		
1	–	–	–	–	–	2	2	1	–	5	–	1	1	–	2	2	2	1
2	3	22	45	2	2	8	4	1	1	14	2	3	2	2	9	12	12	10
3	9	–	–	4	2	6	1	–	3	10	4	5	3	2	14	4	–	3
4	1	–	–	13	2	2	2	–	–	4	3	1	1	–	5	–	–	–
Total	13	22	45	19	6	18	9	2	4	33	9	10	7	4	30	18	14	14

[a] Some metaphases were not complete (14 or 15 chromosomes); single labelled chromosomes or groups of few chromosomes not scored, so that the number of labelled sites in some cases might be underestimated

Fig. 1 a–c. *Alluim cepa*. **a** Feulgen-stained metaphase; *arrows* indicate the pair of satellite-less original nucleolar chromosomes. **b** of 14 chromosomes from slide 18 with four clearly labelled rDNA sites, two at the end of the short arm of the original nu chromosomes (*arrows*) and two at the ends of the short arm of the smallest submetacentric pair of chromosomes (*arrow* **c** Complete metaphase from slide 13 with only two clearly labelled rDNA sites (*arrows*) at the NOR positions of the original nu chromosomes pair. All photos were taken at the same magnification. Bar represents 10 µm

Fig. 2a, b. Top onion strain 124. **a** Fifteen chromosomes from slide 15 with heavy label at the *A. cepa* nucleolar chromosom the end of the short arm of a large chromosome; weaker label at the short arms of a small submetacentric and the satellite *fistulosum* nucleolar chromosome. **b** Sample of labelled chromosomes. From left to right and top to bottom: three weakly la *A. fistulosum* nucleolar chromosomes, two *A. cepa* nucleolar chromosomes, five large *A. cepa* chromosomes, four smaller submetacentr and three larger more metacentric chromosomes



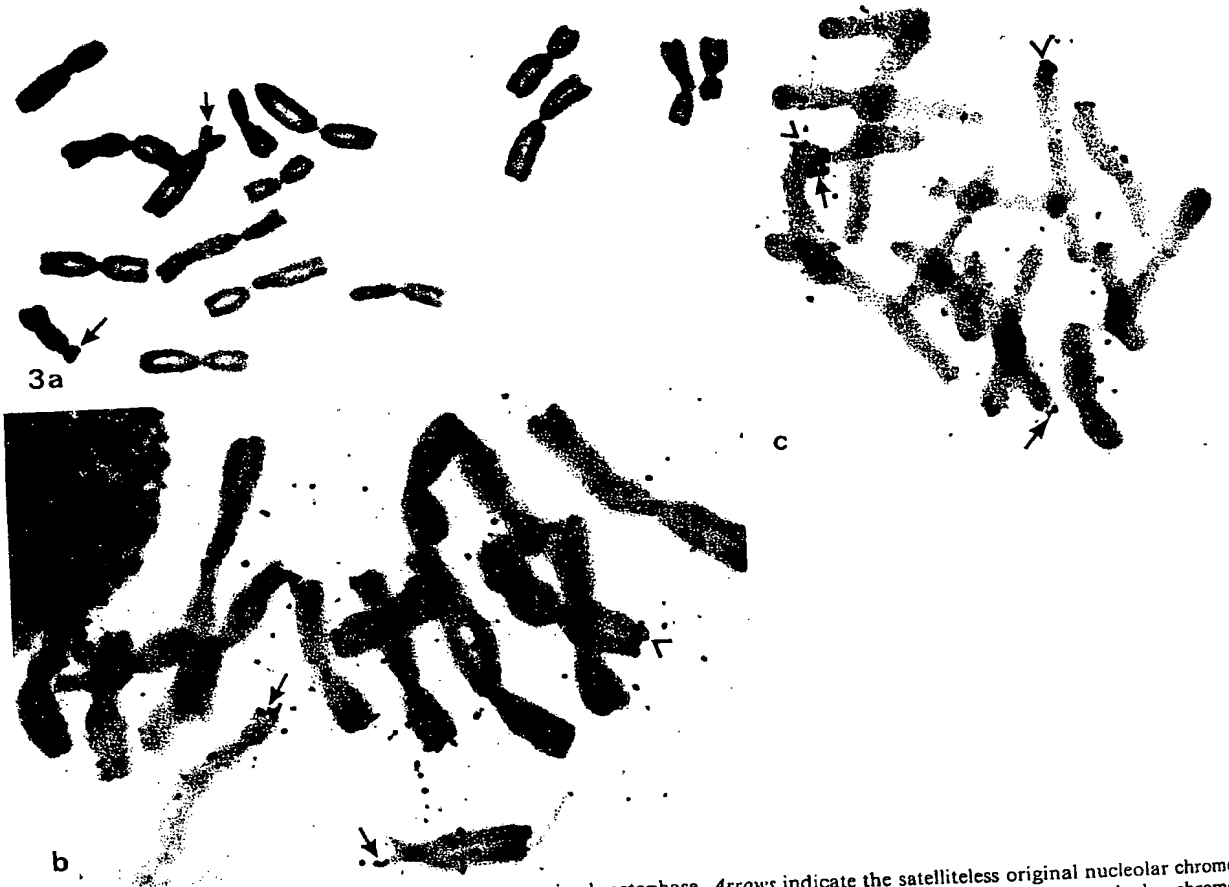


Fig. 3a–c. Top onion strain 437. **a** Complete Feulgen-stained metaphase. *Arrows* indicate the satelliteless original nucleolar chromosomes from *A. fistulosum* (small) and *A. cepa* (large). **b** Metaphase from slide 16 with rDNA label at the original nucleolar chromosomes of *A. cepa* and *A. fistulosum* and at the end of the short arm of a large *A. cepa* chromosome (*arrows*). **c** Similar metaphase from slide 11; additional label at a smaller submetacentric chromosome

silver deposits. This deviation is probably due to differences between the individuals investigated in both sets of experiments.

Top onion strain 437

The four individuals investigated were also characterized by absence from the parental nucleolar chromosomes of any heterochromatic satellites and showed, in principle, the same metaphase labelling patterns as described for strain 124 (Table 1, Fig. 3b, c).

Top onion strain 15

In this strain, the original nucleolar chromosome of *A. fistulosum* retained its satellite while that of the corresponding *A. cepa* chromosome was lacking. Again four slides from different individuals were evaluated.

In one slide no metaphase could be found. Interphases showed one to four labelling centers: most frequently two, sometimes three (Fig. 4c) very rarely one or four. The majority of metaphases in the other slides showed two signals at the NORs of regular nucleolar chromosomes of both species. In this case, however, the *A. fistulosum* NOR was found to be more heavily labelled than the regular *A. cepa* NOR (Fig. 4b). When only one chromosome was labelled the signal was confined to the *A. fistulosum* nucleolar chromosome. A third positive signal occasionally occurred terminally in a large *A. cepa* chromosome (Fig. 4d) or in a chromosome smaller than the usual nucleolar chromosome of *A. cepa* (Table 1). This result differs from that previously obtained by silver staining in that silver deposits were exclusively observed at the regular *A. fistulosum* NOR (Schubert 1984). This is due to differences between the individuals investigated in both experiments, as born out by silver staining of root tip meristems from the same individuals that were used for hybridization experiments. In those cases two, or even three nucleoli per interphase occurred (Fig. 4) instead of only one as observed in individuals used in previous experiments.

We infer from these results that the rDNA sites appear in addition to the regular ones are restricted to heterochromatic ends of short chromosome arms. However, in those cases where a positive hybridization signal appeared in a chromosome larger than the original *A.* nucleolar chromosome, we are not sure whether the

Fig. 4a–e. Top onion strain 15. a Feulgen-stained complete metaphase; *arrows* indicate the nucleolar chromosome of *A. fistulosum* (with satellite) and that of *A. cepa* (without satellite). b Metaphase from slide 9 with rDNA sites at the secondary constriction of the *A. fistulosum* nucleolar chromosome and at the end of the short arm of the *A. cepa* nucleolar chromosome. c Interphase from slide 7 with three clear centers of labelling. d Large *A. cepa* chromosome which somtimes shows additional rDNA label. e Interphase with two silver-stained nucleoli

ling was confined always to one and the same chromosome or whether different chromosomes of the group of the larger *A. cepa* chromosomes were involved. As to the signals in chromosomes smaller than the original nucleolar chromosomes of *A. cepa* (except in the *A. fistulosum* nucleolar chromosome) it remains also an open question whether only the two smallest *A. cepa* chromosome pairs or also the largest chromosomes of *A. fistulosum* are labelled since these chromosomes are rather similar in size and morphology.

Discussion

The results obtained by rDNA hybridization in situ correspond, in principle, to those found after silver staining of NORs and nulceoli in the *Allium* species under investigation.

The following findings argue against an activation-inactivation process of NORs and support the hypothesis of interchromosomal mobility of NORs in *Allium* genomes:

(1) In *A. cepa*, one to four Ag-NORs may occur (Sato 1981) in different individuals. Our in situ hybridization data also revealed two to four labelled chromosomes in different individuals and not always four as would be expected in case of a differential activation or inactivation of NORs at permanent rDNA sites.

(2) In top onion strains, which should have up to three NORs (one from *A. fistulosum* and maximally two from *A. cepa*), one to four rDNA sites were observed and as with $AgNO_3$ staining, there was not only variability between strains and individuals but also between metaphases from the same individual (see Table 1).

(3) At least one of the large *A. cepa* chromosomes, which in the parental species showed neither Ag-NOR staining nor an rDNA hybridization signal, showed silver deposits (Schubert 1984) as well as hybridization signals in some cells of the top onion strains.

(4) With respect to the label observed in chromosomes smaller than the usual *A. cepa* nucleolar chromosome (the *A. fistulosum* nucleolar chromosome excluded) it was not always the same smallest submetacentric *A. cepa* chromosome that was found to be labelled. In addition, the second smallest, more metacentric chromosome of *A. cepa* or possibly one of the largest *A. fistulosum* chromosomes could form the Ag-NOR or exhibit an rDNA hybridization site (Fig. 2b).

Therefore, it seems that NORs of some *Allium* chromosomes are free to jump at least between some preferential chromosomal sites, either by means of adjacent transposable elements or due to the presence of recombination hot spots in the terminal heterochromatin blocks of the short chromosome arms that find homologous sequences inside or proximal to the NORs. Surprisingly, the most pronounced mobility occurs when the original NOR chromo-

somes lost their satellites. No good explanation for this fact is presently available.

A number of data make us believe that mobility of NORs is not confined to *Allium*. Variability of the number of nucleoli in *Crotalaria* (Verma and Raina 1981), the occurrence of additional rDNA with NOR-forming ability in the intercalary heterochromatin of *Drosophila* (Ilyin et al. 1978), and recent data obtained in man point in the same direction. Zakharov et al. (1982) found human individuals with different numbers of Ag-NORs. The chromosomal position of NORs in these individuals exactly corresponded to the rDNA hybridization patterns (Zakharov and Egolina, personal communication). This excludes the possibility that the observed variability was due to activation or inactivation of the rDNA sites in question and receives additional support from the evidence of genetic exchanges between nonhomologous human NORs (Arnheim et al. 1980).

The only alternative explanation of NOR variability would be, according to our opinion, that single orphonlike rDNA copies at different chromosomal sites (Childs et al. 1981) – undetectable by in situ hybridization or by $AgNO_3$ staining – could under certain conditions amplify very quickly to form functional NORs. However, at present we are unaware of any experimental data in support of this assumption.

Ackowledgements. We are very grateful to Dr. S. Tanifuji, for his kindness in providing us with the *Vicia faba* rDNA clone VER17, to Drs. A.F. Zakharov and N.A. Egolina for personal communication of their unpublished results, to Barbara Hildebrandt for excellent technical assistance, and to Prof. R. Rieger for critical reading of the manuscript.

References

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, Zimmer E (1980) Molecular evidence for genetic exchange among ribosomal genes on non-homologous chromosomes in man and apes. Proc Natl Acad Sci USA 77:7323–7527

Childs G, Maxson R, Cohn RH, Kedes L (1981) Orphons: Dispersed genetic elements derived from tandem repetitive genes of eucaryotes. Cell 23:651–663

Ilyin YV, Tchurikov NA, Ananiev EV, Ryskov AP, Yenikolopov GN, Limborska SA, Maleeva NE, Gvozdev VA, Georgiev GP (1978) Studies on the DNA fragments of mammals and Drosophila containing structural genes and adjacent sequences. Cold Spring Habor Symp Quant Biol 42:959–969

Knälmann M, Burger E-C (1979) Degree of relatedness of ribosomal 18/25S RNA of Vicia species and Allium cepa. Cytobiologie 18:422–430

Navashin M (1927) Changes in the number and form of chromosomes as a result of hybridization. Z Zellforsch Mikrosk Anat 6:195–233

Navashin MS (1928) "Amphiplastie" – Eine neue karyologische Erscheinung. Proc Int Conf Genet 5:1148–1152

Nicoloff H, Anastassova-Kristeva M, Rieger R, Künzel G (1979) 'Nucleolar dominance' as observed in barley translocation lines with specifically reconstructed SAT chromosomes. Theor Appl Genet 55:247–251

Rieger R, Nicoloff H, Anastassova-Kristeva M (1979) 'Nucleolar dominance' in interspecific hybrids and translocation lines – A review. Biol Zentralbl 98:385–398

Sato S (1981) Cytological studies on the satellited chromosomes of Allium cepa. Caryologia 34:431–440

Schubert I (1984) Mobile nucleolus organizing regions (NORs) in Allium (Liliaceae s. lat.)? – Inferences from the specificity of silver staining. Plant Syst Evol 144:291–305

Schubert I, Ohle H, Hanelt P (1983) Phylogenetic conclusions from Giemsa banding and NOR staining in top onions (Liliaceae). Plant Syst Evol 143:245–256

Verma RC, Raina SN (1981) Cytogenetics of Crotalaria. V. Supernumerary nucleoli in C. agatiflora (Leguminosae). Genetica 56:75–80

Yakura K, Tanifuji S (1983) Molecular cloning and restriction analysis of Eco-RI fragments of Vicia faba rDNA. Plant Cell Physiol 24:1327–1330

Zakharov AF, Davudov AZ, Benjush VA, Egolina NA (1982) Polymorphisms of Ag-stained nucleolar organizer regions in man. Hum Genet 60:334–339

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Preparation of tomato meiotic pachytene and mitotic metaphase chromosomes suitable for fluorescence *in situ* hybridization (FISH)

Xiao-Bo Zhong, J. Hans de Jong & Pim Zabel

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Fluorescence *in situ* hybridization (FISH) is an increasingly powerful tool with a variety of applications in both basic and applied research. With excellent genetic, cytogenetic and molecular maps available, the tomato genome provides a good model to benefit from the full potential of FISH. Tomato chromosomes at mitotic metaphase are small and not particularly suitable for high-resolution FISH. In contrast, chromosomes at meiotic pachytene are about 15 times longer, and easier to identify by their differences in chromosome arm lengths and chromomere pattern. We have developed a technique for preparing chromosomal spreads of young pollen mother cells at midprophase I which is suitable for FISH. In a first series of experiments, the hybridization patterns of three classes of repetitive DNA sequences were studied in single and multicolour FISH.

Key words: fluorescence *in situ* hybridization, pachytene, metaphase, repetitive sequences, tomato

Introduction

With the advent of the fluorescence *in situ* hybridization (FISH) technique, cytogenetics has become instrumental in analysing the molecular organization of eukaryote chromosomes (for review see Heslop-Harrison 1991, Joos *et al.* 1994). In animal and human systems, multicolour FISH has thus been applied to construct cytogenetic maps showing the positions and order of molecular probes along the chromosomes, including cosmids (Lichter *et al.* 1990, Inazawa *et al.* 1994), YACs (yeast artificial chromosomes) (Selleri *et al.* 1992, Marrone *et al.* 1994, Moir *et al.* 1994) and small, single-copy probes (Heppell-Parton *et al.* 1994, Muleris *et al.*. 1994). As for plants, FISH has seen so far different types of applications (for review see Jiang & Gill 1994), ranging from the detection of alien chromosomes or segments in cereals (Schwarzacher *et al.* 1992a, Mukai *et al.* 1993) and the detection of parental chromosomes in (a)symmetric somatic hybrids (Parokonny *et al.* 1992, Schwarzacher *et al.* 1992b, Wolters *et al.* 1994, Jacobsen *et al.* 1995), to the mapping of repetitive and low-copy DNA sequences in various species (Leitch *et al.* 1991, Maluszynska & Heslop-Harrison 1991, Albini & Schwarzacher 1992, Xu & Earle 1994).

Tomato, with its excellent cytogenetic, genetic and molecular maps (Khush and Rick 1968, Tanksley *et al.* 1992), provides a good model for applications of FISH. In focusing our attention on chromosome 6, we have recently succeeded in integrating the molecular and classical linkage data into a combined map (Weide *et al.* 1993, Van Wordragen *et al.* 1994). With the final goal of developing a cytogenetically based physical map of chromosome 6 that further combines the order of loci from the molecular/genetic linkage map with cytological markers, we have started a molecular cytogenetic analysis of the tomato genome using FISH. As tomato chromosomes at mitotic metaphase are too condensed (2–4.5 μm) for high-resolution physical mapping, such a map should be constructed using chromosomes at pachytene stage exploring their distinct morphology and highly differentiated pattern of euchromatin and heterochromatin (Ramanna & Prakken 1967). In this report, we present a technique for preparing chromosome spreads of young pollen mother cells at midprophase I that is particularly suitable for applications in FISH. As an example, the mapping of three classes of repetitive DNA sequences is described.

Materials and methods

Lycopersicon esculentum cv Cherry was used in all experiments. The following DNA clones were selected for probe labelling: (1) rDNA, an 0.7 kb petunia genomic DNA fragment derived from the 5′ end of the 25S rRNA gene (Van Blokland *et al.* 1994); (2) TGR1, a 162 bp telomere-associated satellite repeat (Ganal *et al.* 1988), localized on most of the distal chromosome ends (Lapitan *et al.* 1989); (3) THG2, a 452-bp repeat member of a large, complex dispersed repeat (Zabel *et al.* 1985).

Young anthers about 3–4 mm long were selected for meiotic chromosome preparations. The stage of development was determined routinely in an acetocarmine squash preparation using a single anther from a flower bud. If at prophase I, the remaining anthers were fixed directly in acetic acid–ethanol (1:3) for 15–30 min, rinsed in deionized water and then in-

X.-B. Zhong and P. Zabel (corresponding author) are at the Department of Molecular Biology, J. Hans de Jong is at the Department of Genetics, Wageningen Agricultural University, Dreijenlaan 3, 6703 HA, Wageningen, The Netherlands. Fax (+31) 8370 83584.

cubated at 37°C for 2–3 h in a mixture of pectolytic enzymes containing 0.3% cytohelicase (Sepracor, France), 0.3% cellulase 'Onozuka' RS (Yakult Honsha, Tokyo, Japan) and 0.3% pectolyase Y-23 (Sigma P3026) in 10 mM citrate buffer, pH 4.5. After two washes in deionized water, the anthers were carefully transferred onto grease-free slides, and the pollen mother cells were dissected out of the anthers into a 1-µl droplet of water using fine-mounted needles, thereby taking care to remove supporting tissue as much as possible. Then 5 µl of 60% acetic acid was added and the pollen mother cells were left for 1–2 min until the cytoplasm became sufficiently clear. A rim of freshly prepared, ice-cold (0°C) fixative was put onto the slide around the droplet containing the meiotic cells. Shortly after the fixative had mixed with the cell suspension, the cells were spread on the slide by adding some more drops of fixative on top of the material. Immediately after this treatment, the slide was briefly immersed in absolute ethanol, and then left to dry. The slides could be used directly for the *in situ* hybridization or were stored at −20°C for up to several months.

Slides with mitotic metaphase complements were obtained from root tip meristems. The root tips from 2- to 4-week-old plants were pretreated in the spindle inhibitor 8-hydroxyquinoline (2 mM) for 2.5 h at 17°C and fixed in acetic acid–ethanol (1:3) fixative for at least 15 min. Further treatments were as described for the meiotic cells, though the enzymatic incubation was limited to 1–2 h in a mixture containing only 0.1% of the three enzymes.

Probe DNA (1 µg) was labelled with either biotin-16-dUTP or digoxigenin-11-dUTP by random primer or nick translation labelling using the protocols of the manufacturer (Boehringer Mannheim, Germany). The *in situ* hybridization protocol was carried out according to Wiegant *et al.* (1991) with some modifications. The slides were pretreated with 100 µg/ml DNAase-free RNAase A in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) at 37°C for 1 h and then washed three times for 5 min in 2 × SSC. Then, the slides were incubated in 5 µg/ml (20 units/ml) pepsin in 0.01 M HCl for 7–10 min at 37°C, washed three times in 2 × SSC for 5 min, treated in 1% (w/v) alkaline formaldehyde (in borate buffer, pH 8.6) for 10 min at room temperature, washed three times in 2 × SSC for 5 min, dehydrated in a graded ethanol series (70%, 90% and 100%), and finally air dried. The hybridization mixture (20 µl per slide, containing 50% formamide, 2 × SSC, 10% sodium dextran sulphate, 50 mM phosphate buffer pH 7.0, 1–2 ng/µl probe DNA and 50–100 ng/µl salmon sperm DNA) was added to the pretreated chromosome preparations and heated to 80°C for 2 min to denature the probe DNA and the chromosomal DNA. *In situ* hybridization was allowed to proceed at 37°C overnight, followed by post-hybridization washes for 3 × 10 min in 50% formamide, 2 × SSC pH 7.0 at 42°C, 10 min in 2 × SSC at room temperature, 3 × 10 min in 0.1 × SSC at 56°C and 10 min in 2 × SSC at room temperature.

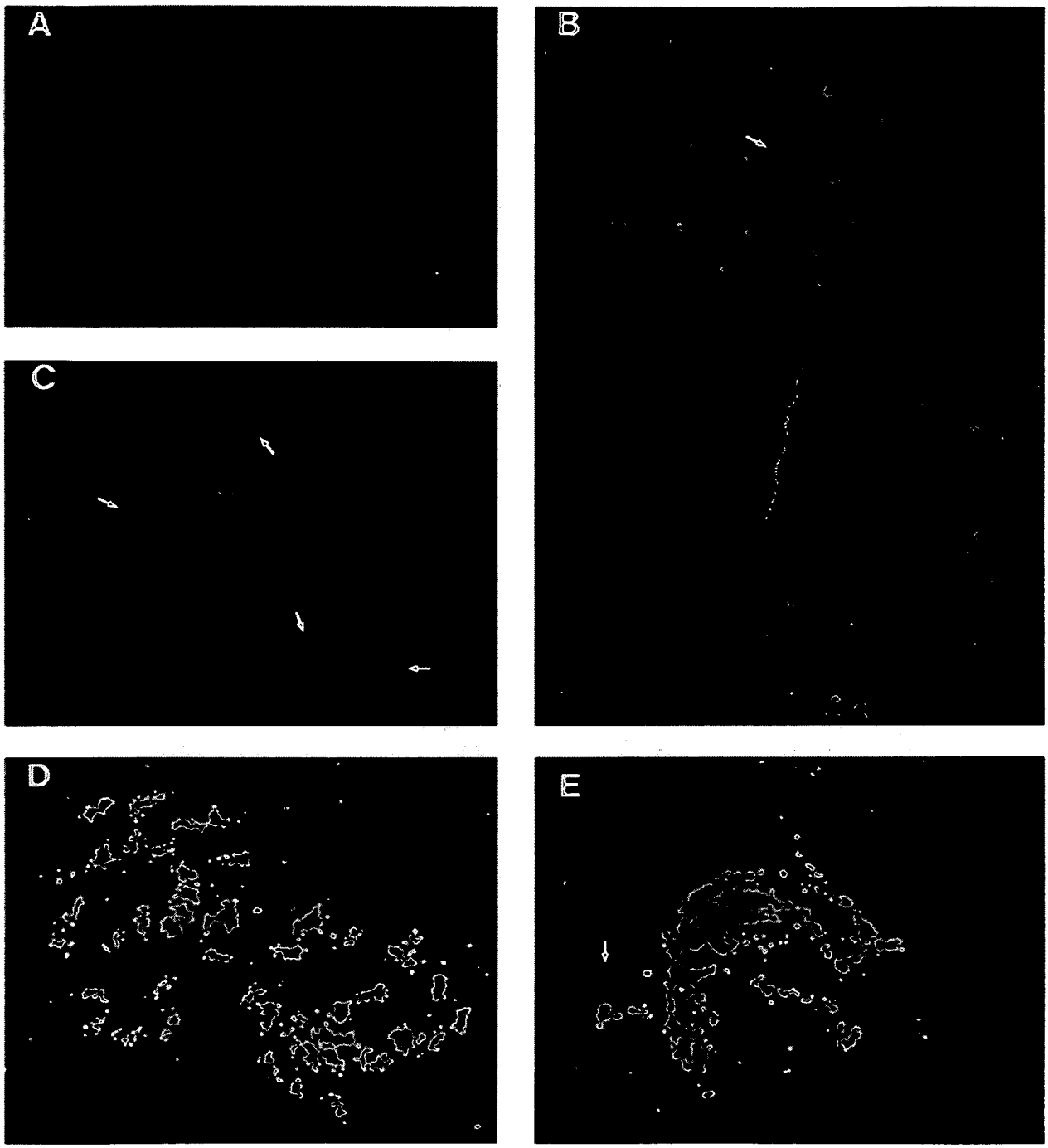
Detection and amplification was according to the manufacturer's protocols (Boehringer Mannheim). Digoxigenin-labelled probes were detected with fluorescein-conjugated anti-digoxigenin antibodies and amplified with fluorescein-conjugated rabbit anti-sheep antibodies (F135, Nordic). Biotin-labelled probes were detected with avidin–Texas red and amplified with biotin-conjugated goat anti-avidin and avidin–Texas red. Chromosomes were counterstained with DAPI (5 µg/ml in McIlvaine buffer, pH 7) or propidium iodide (5 µg/ml in water). The slides were mounted in Vectashield (Vecta Laboratories) antifade mounting. The hybridization signals were studied in a Zeiss Axioplan microscope equipped with Plan Neofluar optics and epifluorescence illumination using the filter sets 01, 09 and 14 for DAPI, FITC and TRITC respectively. Photographs were taken on 400 ISO colour negative film. The images obtained from simultaneous hybridization with two different probes were captured with a high-sensitivity charge-coupled device (CCD) camera (Extended Isis, Photonic Science, UK) or scanned from negatives, merged and pseudocoloured using appropriate image processing software.

Results

Successful localization of DNA sequences on chromosome spreads requires preparations of high quality. This is even more true for meiotic cells in which remnants of thick callose walls and cytoplasm generate higher levels of background and reduced hybridization signals. Therefore, our initial experiments were primarily focused on improving the technique for combining optimal spreading of well-differentiated chromosomes with limited cell damage and clear background. Crucial improvements were achieved by:

- Limiting the fixation time of the anthers to only 15–30 min.
- Prolonging the digestion with highly purified pectolytic enzymes to achieve complete breakdown of the callose walls without any risk of affecting the chromatin integrity.
- Controlled spreading of the cells using ice-cold acetic acid–ethanol fixative. Unlike the traditional

Figure 1. Fluorescence *in situ* hybridization patterns of three repetitive sequences on metaphase and pachytene chromosomes of tomato, *Lycopersicon esculentum* cv. Cherry. **A** FISH of rDNA on pachytene chromosomes. The rDNA probe was labelled with digoxigenin and detected with anti-digoxigenin–FITC. (× 1500). **B** FISH of TGR1 on pachytene chromosomes. The TGR1 probe was labelled with digoxigenin and detected with anti-digoxigenin–FITC. The arrow indicates the large heterochromatin satellite region at the short arm of chromosome 2 (× 1910). **C** Simultaneous FISH of rDNA and TGR1 on metaphase chromosomes. The rDNA probe was labelled with digoxigenin and detected with anti-digoxigenin–FITC (green) and the TGR1 probe was labelled with biotin and detected with avidin–Texas red (red). The three-colour image has been obtained by merging and pseudocolouring the FITC, Texas red and DAPI images using appropriate image-processing software. The arrows indicate the chromosomes without the TGR1 signals at the ends of the long arms (× 1900). The chromosomes in **A–C** were counterstained with DAPI. **D & E** FISH of THG2 on metaphase and pachytene chromosomes respectively. The THG2 probe was labelled with digoxigenin and detected with anti-digoxigenin–FITC. The chromosomes were counterstained with propidium iodide. The arrow in **E** indicates the large heterochromatin satellite region at the short arm of chromosome 2 (**D**, × 1520; **E**, × 1450).



squashing method, cell spreading protocols in tomato generally caused less chromosome loss and damage and resulted in an increase in the number of properly spread and complete meiotic prophase nuclei that were virtually free of cytoplasm.
Briefly washing the slides in absolute ethanol immediately after cell spreading to clear up the background further.

Tomato chromosomes at pachytene are approximately 15 times longer than at mitotic metaphase (Ramanna & Prakken 1976) and exhibit a well-differentiated pattern of eu- and heterochromatin, which, along with chromosome length and centromere position, permits the identification of all 12 pairs. Upon DAPI or propidium iodide counterstaining, this pattern is largely retained, showing prominent fluorescing blocks at the

pericentromeric regions and the telomeres, along with faint fluorescence at the euchromatic parts and the centromeres (see Figure 1A, B & E). The satellite of chromosome 2, which is quite large in the tomato cultivar used, showed a brighter fluorescence, probably reflecting the presence of AT-rich highly repetitive DNA sequences (see arrows in Figure 1B & E).

To establish optimum conditions for FISH, pachytene chromosome preparations were subjected to hybridization with some control probes of known chromosome location. Hybridization with digoxigenin-labelled rDNA, followed by detection with anti-digoxigenin–FITC, revealed a strong and specific green fluorescent signal at the large distal block of the satellite chromosome of chromosome 2 (Figure 1A). The second probe tested was the telomere-associated satellite sequence TGR1, a 162-bp repeat shown to be located on 40 out of the 48 chromosome ends and on some interstitial sites of mitotic metaphase chromosomes (Ganal *et al.* 1988, Lapitan *et al.* 1989). Simultaneous hybridization of biotin-labelled TGR1 and digoxigenin-labelled rDNA probes on metaphase chromosomes showed bright red fluorescent signals of TGR1 at 40 of the 48 chromosomal ends (Figure 1C). No TGR1 signals were detected on chromosome 2 (identified by carrying the green signals of the rDNA probe), whereas two pairs of chromosomes only exhibited signals at their short arms (see arrows in Figure 1C). FISH of digoxigenin-labelled TGR1 on pachytene chromosomes (Figure 1B) revealed 20 out of 24 pachytene bivalent ends with green TGR1 signals. There was a striking difference in size of the TGR1 signals, suggesting a considerable variation in sequence length of this satellite repeat among the chromosomes. The pachytene bivalents not only showed a distribution of distal TGR1 signals similar to that of the metaphase chromosomes (Figure 1C), but they also revealed small TGR1 spots at some interstitial sites.

The 452-bp *Hin*dIII repeat THG2 is a cloned member of a large, complex dispersed repeat family specific for the tomato genome (Zabel *et al.* 1985, Wolters *et al.* 1991). Its distribution pattern was studied in preparations containing spread nuclei from both pollen mother cells at pachytene (Figure 1E) and their accompanying endopolyploid tapetal cells (Figure 1D) using digoxigenin-labelled THG2 probe. Hybridization on the highly condensed metaphase chromosomes indicated that this repetitive sequence occurs on all chromosomes, but that the number of copies differs considerably among the individual chromosomes. Here again, hybridization on pachytene chromosomes showed superior resolution. Signals were mainly confined to the proximal heterochromatin regions.

Discussion

Our focus was primarily on screening current chromosome techniques for their ability to yield high numbers of meiotic nuclei from dissected anthers and to spread their chromosomes without notable loss and damage. We have improved the cell spreading and subsequent treatments in such a way that the characteristic differentiation of euchromatin and heterochromatin segments of pachytene bivalents, as described for carmine- or Feulgen-stained squash preparations, was fully retained in the DAPI- or propidium iodide-stained chromosomes. Such differentiation is indispensable for the identification of all 12 bivalents and, thus, for assigning molecular markers and repetitive sequences by FISH to specific regions of the cytogenetic map.The physical resolution of chromosomes at pachytene stage is, on average, 15 times higher than at metaphase stage, but differs significantly between euchromatic and heterochromatin parts of the chromosomes. Ramanna & Prakken (1967) made a comparison of the mean lengths of these chromatin segments between pachytene chromosomes and the corresponding metaphase chromosomes and found that proximal heterochromatic segments were reduced in length by a factor of 4–5, whereas the euchromatic parts might differ by a factor of 25–30. This implies that such differential contraction should be taken into account when deducing physical distances between markers on the chromosomal DNA. In addition, the more condensed chromatin segments might also be less accessible to probes in FISH experiments.

FISH conditions for both metaphase and pachytene spreads have been established using three different types of repetitive DNA sequences, two of which have known chromosomal location, ribosomal DNA and the telomere-associated repeat TGR1 (Ganal *et al.* 1988, Lapitan *et al.* 1989). In all cases studied, FISH on pachytene spreads was clearly superior in providing signals at a much higher resolution. As for the dispersed repeat probe THG2, for example, FISH on pachytene spreads showed a highly defined dispersion pattern with signals mainly confined to the heterochromatin regions and large stretches of chromosomes devoid of any signal. In contrast, virtually no differentiation in signal distribution was obtained in employing metaphase spreads, indicating that any future high-resolution mapping in tomato should be conducted with chromosomes at meiotic pachytene stage.

As is already common practice in animal and human cytogenetics, FISH offers the possibility to visualize simultaneously multiple targets in a single specimen. In tomato, multicolour FISH would be a powerful tool in constructing integrated chromosome maps. In this regard, the simultaneous detection of two classes of repeats as described in the present paper provides a first step towards this goal.

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References

Albini SM, Schwarzacher T (1992) *In situ* localization of two repetitive DNA sequences to surface-spread pachytene chromosomes of rye. *Genome* **35**: 551–559.

Ganal MW, Lapitan NLV, Tanksley SD (1988) A molecular and cytogenetic survey of major repeated DNA sequences in tomato (*Lycopersicon esculentum*). *Mol Gen Genet* **213**: 262–268.

Heppell-Parton AC, Albertson DG, Fishpool R, Rabbitts PH (1994) Multicolour fluorescence *in situ* hybridization to order small, single copy probes on metaphase chromosomes. *Cytogenet Cell Genet* **66**: 42–47.

Heslop-Harrison JS (1991) The molecular cytogenetics of plants. *J Cell Sci* **100**: 15–21.

Inazawa J, Ariyama T, Tokino T *et al.* (1994) High resolution ordering of DNA markers by multicolour fluorescent *in situ* hybridization of prophase chromosomes. *Cytogenet Cell Genet* **65**: 130–135.

Jacobsen E, de Jong JH, Kamstra SA, van den Berg PMMM, Ramanna MS (1995) Genomic *in situ* hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. *Heredity* **74**: 250–257.

Jiang JM, Gill BS (1994) Nonisotopic *in situ* hybridization and plant genome mapping: the first 10 years. *Genome* **37**: 717–725.

Joos S, Fink TM, Ratsch A, Lichter P (1994) Mapping and chromosome analysis: the potential of fluorescence *in situ* hybridization. *J Biotechnol* **35**: 135–153.

Khush GS, Rick CM (1968) Cytogenetic analysis of the tomato genome by means of induced deficiencies. *Chromosoma* **23**: 452–484.

Lapitan NLV, Ganal MW, Tanksley SD (1989) Somatic chromosome karyotype of tomato based on *in situ* hybridization of the TGR1 satellite repeat. *Genome* **32**: 992–998.

Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. *Genome* **34**: 329–333.

Lichter P, Tang CC, Call K, Hermanson G, Evans GA, Housman D, Ward DC (1990) High resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* **247**: 64–69.

Maluszynska J, Heslop-Harrison JS (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. *Plant J* **1**: 159–166.

Marrone BL, Campbell EW, Anzick SL *et al.* (1994) Mapping of low-frequency chimeric yeast artificial chromosome libraries from human chromosomes 16 and 21 by fluorescence *in situ* hybridization and quantitative image analysis. *Genomics* **21**: 202–207.

Moir DT, Dorman TS, Day JC *et al.* (1994) Toward a physical map of human chromosome 10: isolation of 183 YACs representing 80 loci and regional assignment of 94 YACs by fluorescence *in situ* hybridization. *Genomics* **22**: 1–12.

Mukai Y, Friebe B, Hatchett JH, Yamamoto M, Gill BS (1993) Molecular cytogenetic analysis of radiation-induced wheat–rye terminal and intercalary chromosomal translocations and the detection of rye chromatin specifying resistance to Hessian fly. *Chromosoma* **102**: 88–95.

Muleris M, Apiou A, Olschwang S, Thomas G, Dutrillaux B (1994) Mapping of 18 probes on human chromosome 18 using single and double colour FISH. *Cytogenet Cell Genet* **65**: 82–85.

Parokonny AS, Kenton AY, Gleba YY, Bennett MD (1992) Genome reorganization in *Nicotiana* asymmetric somatic hybrids analysed by *in situ* hybridization. *Plant J* **2**: 863–874.

Ramanna MS, Prakken R (1967) Structure of and homology between pachytene and somatic metaphase chromosomes of the tomato. *Genetica* **38**: 115–133.

Schwarzacher T, Anamthawat-Jonsson K, Harrison GE *et al.*. (1992a) Genomic *in situ* hybridization to identify alien chromosomes and chromosome segments in wheat. *Theor Appl Genet* **84**: 778–786.

Schwarzacher T, Heslop-Harrison JS, Anamthawat-Jonsson K, Finch RA, Bennett MD (1992b) Parental genome separation in reconstructions of somatic and premeiotic metaphase of *Hordeum vulgare* × *H. bulbosum*. *J Cell Sci* **101**: 13–24.

Selleri L, Eubanks JH, Giovannini M *et al.*. (1992) Detection and characterization of chimeric yeast artificial chromosome clones by fluorescent *in situ* suppression hybridization. *Genomics* **14**: 536–541.

Tanksley SD, Ganal MW, Prince JP *et al.* (1992) High density molecular linkage maps of the tomato and potato genomes. *Genetics* **132**: 1141–1160.

Van Blokland R, Van der Geest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J* **6**: 861–877.

Weide R, van Wordragen MF, Lankhorst RK *et al.* (1993) Integration of the classical and molecular linkage maps of tomato chromosome 6. *Genetics* **135**: 1175–1186.

Wiegant J, Ried T, Nederlof P, van der Ploeg M, Tanke HJ, Raap AK (1991) *In situ* hybridization with fluoresceinated DNA. *Nucleic Acids Res* **19**: 3237–3241.

Wolters AMA, Schoenmarkers HCH, van der Meulen-Muisers JJM *et al.* (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. *Theor Appl Genet* **83**: 225–232.

Wolters AMA, Schoenmarkers HCH, Kamstra S *et al.* (1994) Mitotic and meiotic irregularities in somatic hybrids of *Lycopersicon esculentum* and *Solanum tuberosum*. *Genome* **37**: 726–735.

Van Wordragen MF, Weide R, Liharska T *et al.* (1994) Genetic and molecular organization of the short arm and pericentromeric region of tomato chromosome 6. *Euphytica* **79**: 169–174.

Xu J, Earle ED (1994) Direct and sensitive fluorescence *in situ* hybridization of 45s rDNA on tomato chromosomes. *Genome* **37**: 1062–1065.

Zabel P, Meyer D, van de Stolpe O *et al.* (1985) Towards the construction of artificial chromosomes for tomato. In: van Vloten-Doting L, Groot GSP, Hall TC, eds. *Molecular Form and Function of the Plant Genome*, NATO ASI series A, *Life Sciences*, Vol. 83, pp 609–624.

Characterization of heterochromatic regions in 'Trovita' orange (*Citrus sinensis* Osbeck) chromosomes by the fluorescent staining and FISH methods

Tomoki Matsuyama, Tomoya Akihama, Yuji Ito, Mitsuo Omura, and Kiichi Fukui

Abstract: CMA/DAPI double staining and FISH methods using rDNA and telomere specific repeated sequences as probes were applied to characterize the large amount of heterochromatin in the telomeric regions of 'Trovita' orange (*Citrus sinensis* Osbeck) chromosomes. As a result, all the heterochromatic regions were CMA+/DAPI−, and thus were GC rich. Three CMA+/DAPI− heterochromatic regions were positive to the 26S rDNA probe. Telomere arrays consisting of $T_m(A)G_n$ were detected on the extreme ends of each chromosome and most of the CMA+/DAPI− heterochromatic regions were ordered close inside the telomere-specific repeated sequences. The data obtained in this study will be useful for karyotyping, systematics, and molecular analysis of orange chromosomes.

Key words: *Citrus*, rDNA, telomere repeated sequence, FISH, CMA/DAPI staining, heterochromatin.

Résumé : La double coloration au CMA/DAPI et la technique FISH, utilisant l'ADNr et les séquences répétées télomériques comme sondes, ont été employées afin de caractériser l'abondante hétérochromatine située dans les régions télomériques des chromosomes de l'oranger 'Trovita' (*Citrus sinensis* Osbeck). Ces travaux ont révélé que ces régions hétérochromatiques sont CMA+/DAPI− et s'avèrent ainsi riches en GC. Trois régions hétérochromatiques CMA+/DAPI− hybridaient positivement avec la sonde d'ADNr 26S. Des séquences télomériques de type $T_m(A)G_n$ ont été détectées aux extrémités de chaque chromosome et la plupart des régions hétérochromatiques CMA+/DAPI− étaient proches et tout juste internes aux séquences répétées télomériques. Les données obtenues seront utiles en vue d'études caryotypiques et de systématique ainsi que pour des analyses moléculaires des chromosomes de l'oranger.

Mots clés : *Citrus*, ADNr, séquence télomérique répétée, FISH, coloration au CMA/DAPI, hétérochromatine.
[Traduit par la Rédaction]

Introduction

For the systematics and genome analysis of *Citrus*, the analysis of chromosomes is very important. Recently, Guerra (1993) reported the variability and heterozygosity of *Citrus* karyotypes and also noted large heterochromatic regions that were enhanced by CMA staining and quenched by DAPI (CMA+/DAPI− heterochromatins) in the six *Citrus* species.

Some of the CMA+/DAPI− heterochromatins were often observed as stretched structures, which were regarded as the secondary constrictions. It is known that the 17S–5.8S–25S ribosomal RNA gene (45S rDNA) and 5S rDNA clusters are separately present in rice and barley genomes, and that such regions correspond to secondary constrictions or the NOR (nucleolus organizing region; Leitch and Heslop-Harrison 1992; Fukui et al. 1994; Ohmido and Fukui 1995). Therefore, we designed primers and developed a probe according to the 26S rDNA sequences reported by Kolosha and Fodor (1990). Telomere-specific repeated sequences (TRS) were used to order the CMA+/DAPI− heterochromatins on terminal regions of each chromosome as well.

We present here detailed information on the CMA+/DAPI− heterochromatin of *Citrus*, characterized using the double-staining method of the guanine–cytosine (GC)-specific fluorochrome CMA and the adenine–thymine (AT)-specific fluorochrome DAPI and a fluorescent in situ hybridization (FISH) method.

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T. Matsuyama[1] **and T. Akihama.**[2] Faculty of Agriculture, Meiji University, Kawasaki 214, Japan.
Y. Ito.[3] Fruit Tree Research Station, Morioka Branch, Morioka 020-01, Japan.
M. Omura. Fruit Tree Research Station, Okitsu Branch, Shimizu 424-02, Japan.
K. Fukui. Hokuriku National Agricultural Experiment Station, Joetsu 943-01, Japan.

[1] Present address: Plant Functions Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan.
[2] Author to whom all correspondence should be addressed.
[3] Present address: Institute of Radiation Breeding, National Institute of Agrobiological Resources, Ohmiya-machi, Naka-gun, Ibaraki 319-22, Japan.

Fig. 1. Double staining of 'Trovita' chromosomes with CMA(A) and DAPI(B). Arrowheads indicate the CMA+/DAPI− heterochromatins. Large arrowheads indicate the stretched segments. Scale bar = 4 μm. **Fig. 3.** In situ hybridization to interphase nuclei (A) and prometaphase chromosomes (B) probed by the 26S rDNA clone (pTM 26) and counterstained with PI. **Fig. 4.** In situ hybridization to prometaphase chromosomes probed by the synthetic telomere probe. (A) Stained with PI. (B) Hybridization signals.

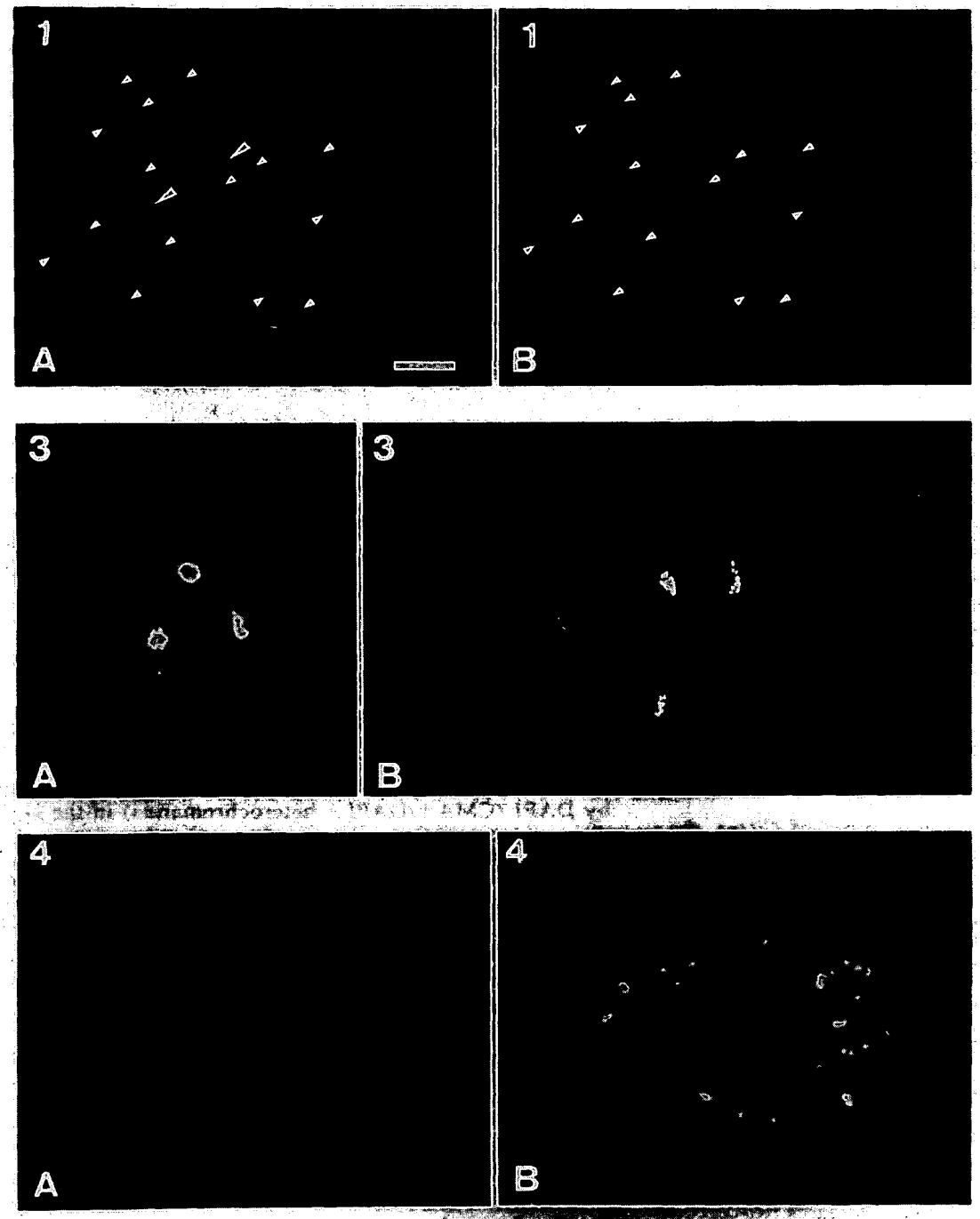
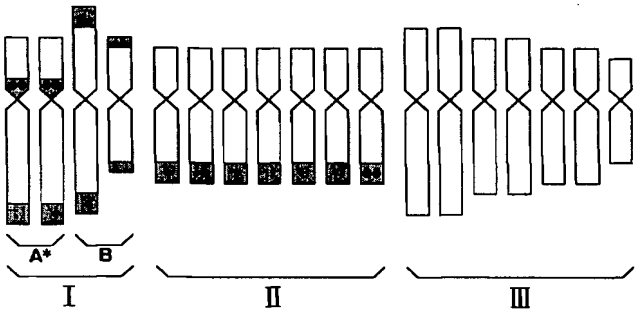


Fig. 2. Idiogram of 'Trovita' chromosomes with CMA+/DAPI− heterochromatin and 26S rDNA clusters. ▨, CMA+/DAPI− heterochromatin; ●, hybridization signal of 26S rDNA.
*The centromeric positions of type I-A were decided upon according to Guerra's data.



Materials and methods

Plant materials

'Trovita' orange (*Citrus sinensis* Osbeck) and 'Kiyomi' tangor (a hybrid between 'Miyagawa wase' unshiu (*Citrus unshiu* Marc.) and 'Trovita'), which were cultivated at the Okitsu branch of the Fruit Tree Research Station, Japan, were used as the source of seeds and leaf DNA in the experiments.

Cytological procedure

Possible nucellar embryos of 'Trovita' were germinated in petri dishes at 27°C in the dark after removing the seed coat. The chromosome samples were prepared by the method of Ito et al. (1992) with minor modification according to Fukui and Iijima (1991) as follows: (*i*) root tips were pretreated in a 2 mM 8-hydroxyquinoline solution at 15°C for 4 h, and (*ii*) enzymatic maceration was performed with a solution containing 2% (w/v) Cellulase Onozuka RS, 0.3% Macerozyme R-200 (Yakult), 1.5% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C for 60–90 min.

Chromosomes were stained with 0.1 mg/mL CMA and 0.2 μg/mL DAPI solutions according to Hizume et al. (1989) and observed under a fluorescence microscope (Axiophot, Zeiss) with the B-light and uv excitation filters.

Probe preparation

45S rDNA probe

Oligonucleotide primers were designed based on the 26S rDNA sequence of *Citrus limon* (Kolosha and Fodor 1990), and the 1.2 kilobase pair (kbp) coding region of 26S rDNA was amplified by the PCR method using the genomic DNA of 'Kiyomi' tangor isolated by the method of Dellaporta et al. (1983). The PCR products were cloned into pCR1000 (TA cloning kit, Invitrogen), and one of the clones (pTM26) was confirmed as having 89.7% homology with the 26S rDNA sequence of *C. limon* in both sides (903 base pairs (bp)).

Telomere probe

The telomere sequences were amplified using the oligonucleotide primers $(5'\text{-TTTAGGG-}3')_3$ and $(5'\text{-CCCTAAA-}3')_3$ without template DNA (Cox et al. 1993). The amplified DNA was high molecular weight (ca. 0.5–10 kbp) and was biotinylated by nick translation.

FISH

FISH and signal detection were carried out according to Fukui et al. (1994). The hybridization mixture consisted of 50 or 25% formamide, 100 μg/mL sonicated herring sperm DNA, and 7.5 μg/mL labelled probe – 2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate). After overnight hybridization at 37°C, the slides were washed with 2× SSC, 50 or 30% formamide – 2× SSC, 2× SSC, and 4× SSC at 42°C. FITC (fluorescein isothiocyanate) signals were detected by the avidin–FITC conjugate and visualized using a B-light excitation filter. Chromosomal DNA was counterstained with 0.5 μg/mL propidium iodide (PI) and visualized using a G-light excitation filter.

Results and discussion

Karyotype and heterochromatic patterns of 'Trovita'

Phase-contrast microscopy revealed that some of the *Citrus* prometaphase chromosomes have large blocks of heterochromatin on one or both telomeric sites. Although such heterochromatic blocks are also observed in maize (Peacock et al. 1981) and *Tradescantia commelinoides* Schultes. fil. (Cox et al. 1993), not as many are found in *Citrus* (Figs. 1A and 1B, arrowheads). They showed CMA+/DAPI− fluorescence, suggesting they were GC-rich sequences.

We classified the 18 somatic chromosomes of 'Trovita' into three types, I, II, and III, on the basis of the number of CMA+/DAPI− heterochromatic regions, with a further subdivision of type I into subtypes I-A and I-B based on the position of the CMA+/DAPI− heterochromatic regions on the chromosomes, as follows:

Type	Observation
I-A	Two chromosomes with the heterochromatic regions in the interstitial regions and the telomeric regions
I-B	Two median* chromosomes with the heterochromatic regions in both the telomeric regions of the short and long arms
II	Seven median* chromosomes with the heterochromatic regions at the telomeric regions of the long arms
III	Seven median* chromosomes with no heterochromatic regions in either chromosomal arm

*The position of the centromere is expressed as median owing to the value of the arm ratio being 1.0 to 1.7, according to Levan et al. (1964).

Two and one CMA+/DAPI− heterochromatic regions in types I-A and II, respectively, were often stretched (Fig. 1A, large arrowheads). The idiogram of 'Trovita' chromosomes is summarized in Fig. 2.

Types I-A and I-B correspond to B and C, respectively, of Guerra's classification (1993). The observation in the present study seemed basically the same as Guerra's observation of sweet orange, although small telomeric CMA+/DAPI− heterochromatins were not detected during our observations. This may be caused by the difference in fluorochrome concentration.

FISH analysis probed with rDNA

After FISH using 26S rDNA as the probe, three strong signals were observed both on the interphase nuclei (Fig. 3A) and on prometaphase chromosomes (Fig. 3B).

Two of these were located on the proximal CMA+/DAPI− heterochromatin of type I-A and one was located on the telomeric CMA+/DAPI− heterochromatin of type II (Fig. 2). When the rice rDNA clone that covered most of the coding regions of 45S rDNA and the flanking spacer regions was used as a probe to improve the signal strength, six signals, consisting of three weak signals and three strong signals, were detected on the interphase nuclei (data not shown). As the NORs were often observed in the stretched condition in a nucleus, they may show more than one signal. One of the type I-A CMA+/DAPI− heterochromatic regions was always stretched at the prometaphase stage. This suggests differences in transcriptional activity among the three rDNA clusters.

FISH analysis probed with TRS

The formamide concentration in the hybridization mixture and washing solution was lowered to 25–30% to enhance the faint signals found under the usual hybridization and washing conditions that permit more than 80% homology. As a result, the doublet signals at the extreme end of most of the chromosomes could be detected and thus the outside of all the CMA+/DAPI− heterochromatins (Figs. 4A, and 4B).

Several variations have already been reported in the telomere-specific tandem array: TTGGGG in *Tetrahymena*, TG_{1-3} in yeast, TTAGG in silkworm, TTTAGGG in *Arabidopsis*, and TTAGGG in human chromosomes, and thus it was interesting to analyze the sequences of *Citrus* chromosome telomeres (Blackburn and Szostak 1984; Okazaki et al. 1993; Richards and Ausbel 1988; Moyzis et al. 1988). It is known that the ends of *Drosophila* chromosomes are composed of a retrotransposon instead of the $T_m(A)G_n$ sequences (Okazaki et al. 1993; Levis et al. 1993). It was suggested that a kind of retrotransposon might also be located on the ends of *Citrus* chromosomes, since the heterochromatins dominated just the terminal regions in several *Citrus* chromosomes, but actually, $T_m(A)G_n$ sequences were located there, although the homology with the $(TTTAGGG)_n$ sequence of *Arabidopsis* was low.

These results show the exact ordering of the telomeric CMA+/DAPI− heterochromatins and TRS. The exact localization of heterochromatin on the chromosomes would serve in the isolation of RFLP markers of the telomeric regions, using recent techniques of laser dissection and recovery of DNA from chromosome fragments (Fukui et al. 1992, 1995).

The *Citrus* karyotype information is useful for further research of repeated sequences at the molecular level. At the same time, information gained at the molecular level would be used to identify the chromosomes completely, since they are small (~0.4 μm) and most of them are similar in morphology.

The analysis of *Citrus* heterochromatic regions has significant meaning, as there are few examples that conserve a large number of the CMA+/DAPI− heterochromatic regions on the chromosomes among plant species. In addition, "three" large rDNA clusters on the CMA+/DAPI− heterochromatin pose new questions concerning cytological interpretation in *Citrus* speciation. The odd number of these regions is not common in plants. Although the reason has not been determined, it may be important to elucidate the cytoevolutionary events in *Citrus*, including the basic chromosome number, which has been hypothesized as $x = 3$, by Agarwal (1987*a*, 1987*b*), or $x = 18$, by Stace et al. (1993).

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References

Agarwal, P.K. 1987*a*. Cytogenetical investigations in Rutaceae I. Meiotic studies in four *Citrus* species of hybrid origin. Cytologia (Tokyo), **52**: 753–756.

Agarwal, P.K. 1987*b*. Cytogenetical investigations in Rutaceae II. Meiotic studies in three intergeneric hybrids of *Citrus*. Cytologia (Tokyo), **52**: 757–760.

Blackburn, E.H., and Szostak, J.W. 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. **53**: 163–196.

Cox, A.V., Bennett, S.T., Parokonny, A.S., Kenton, A., Callimassia, M.A., and Bennett, M.D. 1993. Comparison of plant telomere locations using a PCR-generated synthetic probe. Ann. Bot. **72**: 239–247.

Dellaporta, S.L., Wood, J., and Hicks, J.B. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. **1**: 19–21.

Fukui, K., and Iijima, K. 1991. Somatic chromosome map of rice by imaging methods. Theor. Appl. Genet. **81**: 597–605.

Fukui, K., Minezawa, M., Kamisugi, Y., Ohmido, N., Ishikawa, M., Yanagisawa, T., Fujishita, M., and Sakai, F. 1992. Microdissection of plant chromosomes by argon ion laser beam. Theor. Appl. Genet. **84**: 787–791.

Fukui, K., Ohmido, N., and Khush, G.S. 1994. Variability in rDNA loci in the genus *Oryza* detected through fluorescence *in situ* hybridization. Theor. Appl. Genet. **87**: 893–899.

Fukui, K., Nomiya, A., Nishiguchi, M., and Fujishita, M. 1995. Application of laser optics to plant cell and molecular biology. Zool. Stud. **34**(Suppl. I): 35–37.

Guerra, M. 1993. Cytogenetics of *Rutaceae*. V. High chromosomal variability in *Citrus* species revealed by CMA/DAPI staining. Heredity, **71**: 234–241.

Hizume, M., Ohgiku, A., and Tanaka, A. 1989. Chromosome banding in the genus *Pinus*. II. Interspecific variation of fluorescent banding patterns in *P. densiflora* and *P. thunbergii*. Bot. Mag. Tokyo, **102**: 25–36.

Ito, Y., Omura, M., Nesumi, H., and Yoshida, T. 1992. Improvement of preparation and observation methods for *Citrus* chromosomes. Bull. Fruit Tree Res. Stn. (Kaju Shikenjo Hokolu), **23**: 57–66.

Kolosha, V.O., and Fodor, I. 1990. Nucleotide sequence of *Citrus limon* 26S rRNA gene and secondary structure model of its RNA. Plant Mol. Biol. **14**: 147–161.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by *in situ* hybridization. Genome, **35**: 1013–1018.

Levan, A., Fradge, K., and Sandberg, A.A. 1964. Nomenclature of centromeric position of chromosomes. Hereditas, **52**: 201–220.

Levis, R.W., Ganesan, R., Houtchens, K., Tolar, L.A., and Sheen, F.-M. 1993. Transposons in place of telomeric repeats at a Drosophila telomere. Cell, **75**: 1083–1093.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L., and

Wu, J.-R. 1988. A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. U.S.A. **85**: 6622–6626.

Ohmido, N., and Fukui, K. 1995. Cytological studies of African cultivated rice, *Oryza glaberrima*. Theor. Appl. Genet. **91**: 212–217.

Okazaki, S., Tsuchida, K., Maekawa, H., Ishikawa, H., and Fujiwara, H. 1993. Identification of a pentanucleotide telomeric sequence, $(TTAGG)_n$, in the silkworm *Bombyx mori* and in other insects. Mol. Cell. Biol. **13**: 1424–1432.

Peacock, W.J., Dennis, E.S., Rhoades, M.M., and Pryor, A.J. 1981. Highly repeated DNA sequence limited to knob heterochromatin in maize. Proc. Natl. Acad. Sci. U.S.A. **78**: 4490–4494.

Richards, E.J., and Ausubel, F.M. 1988. Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. Cell, **53**: 127–136.

Stace, H.M., Armstrong, J.A., and James, S.H. 1993. Cytoevolutionary patterns in *Rutaceae*. Plant Syst. Evol. **187**: 1–28.

Chromosoma (1995) 104:39–43

CHROMOSOMA

Floral chromosomes of *Arabidopsis thaliana* for detecting low-copy DNA sequences by fluorescence in situ hybridization

M. Murata, F. Motoyoshi

Research Institute for Bioresources, Okayama University, Kurashiki 710, Japan

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Abstract. Cosmid and plasmid clones containing 11 kb, or more, of genomic DNA sequences were mapped with high efficiencies using fluorescence in situ hybridization (FISH) to mitotic metaphase chromosomes prepared from floral tissues of *Arabidopsis thaliana*. The chromosomal locations were correlated with the map positions determined by RFLP (restriction fragment length polymorphism) analyses. Almost no signals were detected on the chromosomes of root meristematic tissues when FISH was performed with the same clones as probes. This discrepancy in efficiency of detection is possibly caused by the differences in chromatin structure between the root meristematic tissues and the floral tissues.

Introduction

The plant *Arabidopsis thaliana* is a model for molecular biological and genetical studies. However, compared with other model organisms such as *Drosophila*, mouse, and human, mapping of the genes on the chromosomes in situ has been very limited. This is mainly due to the extremely small size of the mitotic chromosomes. Recently, repetitive sequences have been successfully mapped by FISH (fluorescence in situ hybridization) (Murata et al. 1990, 1994; Maluszynska and Heslop-Harrison 1991), but no low-copy sequences have been mapped on the *Arabidopsis* chromosomes. Although some successes in chromosome mapping of single- or low-copy sequences have been reported in plant species with relatively large chromosomes (Ambros et al. 1986; Mouras et al. 1987; Shen et al. 1987; Huang et al. 1988; Gustafson et al. 1990), the efficiency of detection was not necessarily high, and the techniques have not been widely adopted for other plant species or even for the same species with other probes.

Here we describe a very reliable FISH technique to map cosmid and plasmid clones prepared on *Arabidopsis* chromosomes, from floral tissues of young buds, and also discuss the influence of chromatin structure on the efficiency of hybridization in plant chromosomes.

Materials and methods

Plant materials. A wild ecotype, Columbia of *A. thaliana* (L.) Heynh. was used in this study.

DNA probes and labeling. Cosmid and plasmid clones (Table 1) were used as probes for FISH. The cosmid clones that have been isolated from *A. thaliana* ecotype Columbia (Nam et al. 1989), were supplied by the ABRC (Arabidopsis Biological Resource Center at Ohio State University). Plasmid clone pYK205 is a subclone of λ235 (Chang et al. 1988), containing an 11 kb EcoRI insert in pBluescript(+)KS. This was a gift from Dr. Y. Komeda, Hokkaido University. The cosmid and plasmid DNA were isolated by the alkaline lysis method, purified by CsCl gradient centrifugation (Maniatis et al. 1982), and labeled with biotin-14-dATP using a nick translation kit (BioNick, Gibco BRL). After ethanol precipitation, the labeled DNA was resuspended in 20 μl of deionized formamide.

Chromosome preparation. Chromosome preparations for FISH were made from root meristematic tissue and also from floral tissue of young buds. The methods were essentially the same as described by Murata et al. (1992, 1994) except for the preparation of flower chromosomes. To make chromosome preparations from floral tissues, four sepals were dissected out from each young bud (1.5–2 mm in length) under a dissecting microscope. The buds without sepals were transferred into a 1.5 ml microtube with 1 ml distilled water, and kept at 0°C in iced water for 24 h to allow accumulation of metaphase cells. The buds were then fixed in acetic alcohol (3:1, 99% methanol:glacial acetic acid), and stored at −20°C until slide preparations were made.

After rinsing well with distilled water, the fixed buds were digested with an enzyme solution containing 2% (w/v) cellulase Onozuka R10 (Kinki Yakult) and 20% (v/v) pectinase (Sigma). After 1.5 h incubation at 30°C, tissues were suspended with a micropipet. The suspended cells were rinsed with distilled water, and

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Correspondence to: M. Murata

Table 1. Cosmid and plasmid clones used as probes for fluorescence in situ hybridization (FISH) in this study

Clone number	Original number	Chromosome	Map position[a]	Approximate size of insert (kb)
CD2-67[a]	g6838[b]	1	109.4	36
CD2-68[a]	g6842[b]	2	58.5	39
CD2-4[a]	g2440[b]	3	39.3	30
CD2-43[a]	g4539[b]	4	49.8	33
CD2-29[a]	g4028[b]	5	57.7	29
pYK205	λ235[c]	1	43.9	11

[a] ABRC code (1994)
[b] Goodman's code (Nam et al. 1989)
[c] Meyerowitz's code (Chang et al. 1988)

fixed again. After three changes of fresh fixative, the fixed cells were dropped onto wet, cold slides, and flame-dried. The slides were then stained with 4% (v/v) Giemsa solution diluted with 1/15 M phosphate buffer, pH 6.8 for 20–30 min, and examined with a microscope.

Fluorescence in situ hybridization (FISH). The slides were destained for 2 min with 45% (v/v) acetic acid, and dehydrated through an ethanol series (70%-85%-99%, by vol.) for 5 min each. The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 4 × SSC (2 × SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7.0) and 1 μg/μl each of salmon sperm DNA and yeast tRNA, and 15 ng/μl biotinylated DNA. Fifteen microliters of hybridization mixture was placed on a slide, covered with a coverslip (40 × 24 mm), and sealed with rubber cement. The slides were denatured at 78°C on a hot plate for 2 min. Following hybridization at 37°C overnight, the slides were washed with 50% (v/v) formamide in 2 × SSC at 37°C twice for 10 min, and 2 × SSC three times for 10 min at room temperature. After blocking with 3% (w/v) BSA (bovine serum albumin) in 4 × SSC and 0.05% (w/w) Tween 20, 50 μg/ml FITC (fluorescein isothiocyanate)-avidin conjugate (Boehringer Mannheim) in blocking solution was applied. The slides were mounted with antifading solution (Johnson and Araujo 1981) containing 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and 1 μg/ml PI (propidium iodide). The DAPI- and PI-stained chromosomes with FITC signals were photographed with 400 ASA/ISO color positive film (Fujichrome Provia) under a fluorescent microscope equipped with UV- and B-excitation filters (Zeiss Axioscope), or fluorescent images were obtained with a laser scanning confocal microscope (BioRad MRC 600).

Results

Mitotic chromosomes in flowers

In higher plants, preparations of mitotic metaphase chromosomes are generally made from root meristematic tissues. However, seeds of *A. thaliana* are very small, and their germinating roots are very thin, and this makes it difficult to collect root-tips for the preparation of chromosomes. At least ten germinating seeds were needed to make one air-dried protoplast preparation. The flowers of young buds are also small, but it was not difficult to collect a large number of young buds from an individual plant. In a young flower, dividing cells were observed particularly in the pistil (style and ovule) and petals, and their numbers were similar or greater than those in root meristems (Fig. 1). In the preparations, some mitotic

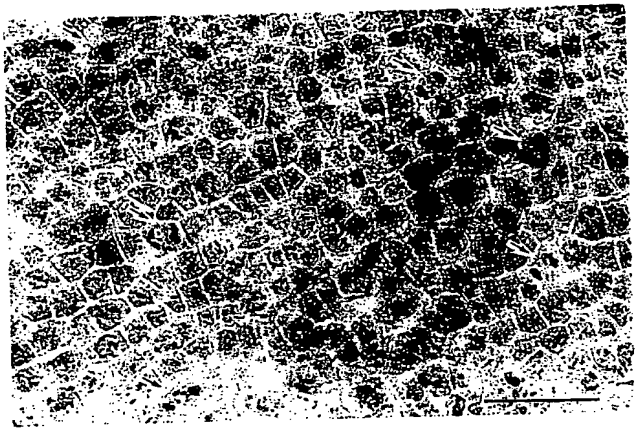

Fig. 1. Cells of young flower petals of *Arabidopsis thaliana* stained with 0.8% acetocarmine. *Arrows* indicate dividing cells. Bar represents 10 μm

prometaphase chromosomes had a unique extended morphology that is not found in root meristematic tissues (Fig. 2). After cold treatment, however, condensed and compact chromosomes were observed as in the root meristems (Fig. 3).

Efficiency of detection of hybridization signals

FISH with biotinylated cosmid and plasmid DNA as probes was first performed on chromosome preparations from root meristematic tissues of germinating seeds. However, almost no signals were detected in any chromosomes of the mitotic metaphase cells (Table 2). In contrast, when the same clones were hybridized to chromosome preparations from young flowers, both homologous chromosomes in almost all metaphase cells showed very distinct double-dot signals (Fig. 4). The efficiency of detection of the double dots on both or either of the homologous chromosomes was 85%–100% (Table 2), and was not significantly affected by the insert size of the cosmid clones between 29 and 39 kb. A slight reduction in efficiency was found for plasmid pYK205 which contains an 11 kb insert (Table 2).

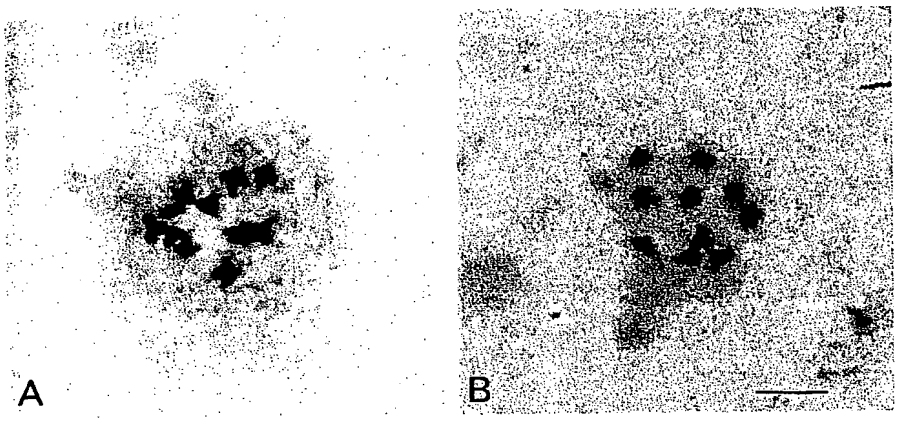

Fig. 2A, B. Mitotic prometaphase cells of *A. thaliana* prepared from young flower buds. Bar represents 2 μm

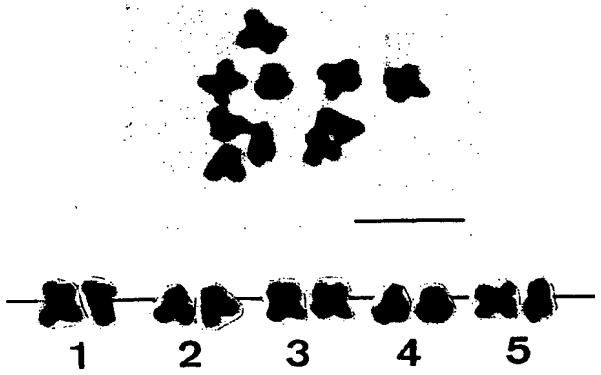

Fig. 3. A mitotic metaphase cell of *A. thaliana* prepared from cold-treated young flower buds, and its karyotype. Bar represents 2 μm

Chromosomal locations of cosmid and plasmid clones

A. thaliana has ten chromosomes in its genome, consisting of three pairs of metacentrics (chromosomes 1, 3, and 5) and two pairs of acrocentrics (chromosomes 2 and 4) (Fig. 3). Although identification of all chromosome components is not easy, each probe showed up only on a specific pair of chromosomes, and the position was almost identical in all cells analyzed. Cosmid clone CD2-67 maps at 109.4 on chromosome 1 (Table 1). This map location was supported by the present FISH, which showed signals at one terminal end of a pair of metacentric chromosomes (Fig. 4A). Similarly, CD2-4 and -29, whose map positions are 39.3 on chromosome 3 and 57.7 on chromosome 5, respectively, were localized on the middle of the long arm of a pair of metacentric chromosomes (Fig. 4C,E). CD2-68 and -43 were mapped on the long arms of acrocentrics, probably chromosomes 2 and 4, respectively (Fig. 4B,D). An insert of pYK205

Table 2. Efficiency of detection of FISH with six different probes in mitotic metaphase chromosomes prepared from flower and root tissues

Clone used	Tissue for chromosome preparation	No. of cells analyzed	No. of cells with signals of				% of cells with double-dot signals
			2D[a]	1D1S[b]	1D[c]	Others[d]	
CD2-67	Flower	20	19	1	0	0	100
	Root	20	0	1	0	19	5
CD2-68	Flower	20	18	2	0	0	100
	Root	20	0	0	0	20	0
CD2-4	Flower	20	18	1	1	0	100
	Root	20	0	0	1	19	5
CD2-43	Flower	20	19	1	0	0	100
	Root	20	0	0	1	19	5
CD2-29	Flower	20	17	2	0	1	95
	Root	20	0	0	0	20	0
pYK205	Flower	20	14	1	2	3	85
	Root	20	0	0	0	20	0

[a] Double-dot signals on both homologous chromosomes
[b] A double-dot signal on one homologue and a single-dot on the other
[c] A double-dot signal on one homologue and no signal on the other
[d] Including 2S (single-dot signals on both homologues), 1S (a single-dot signal on one homologue and none on the other), and 0 (no signal on either homologue)

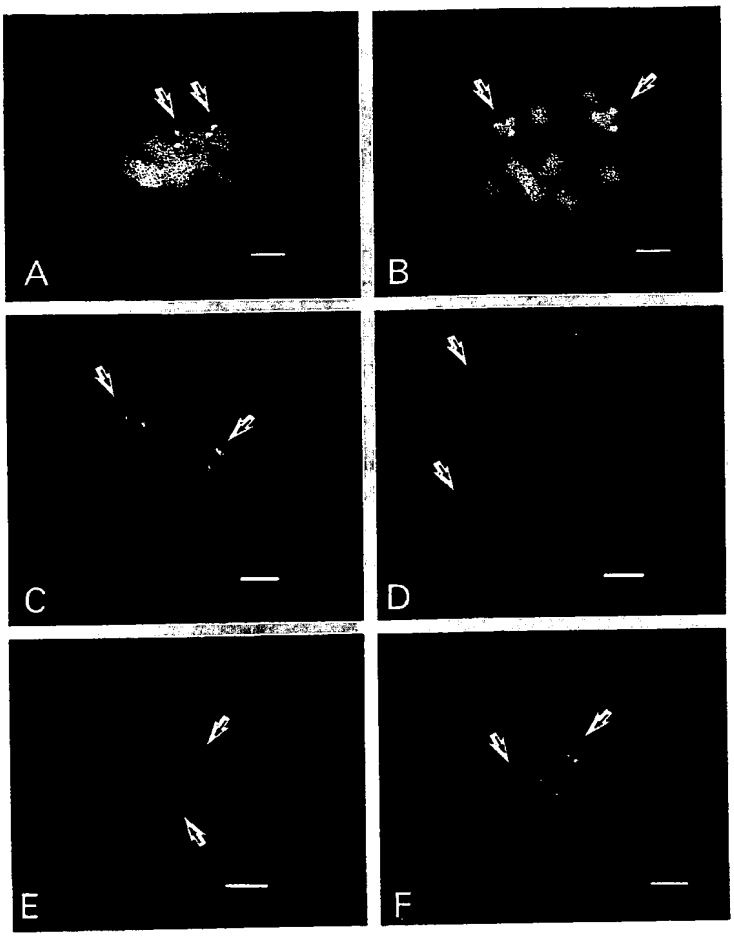

Fig. 4A–F. Propidium iodide (PI)-stained mitotic metaphase chromosomes of *A. thaliana*, showing fluorescein isothiocyanate (FITC) signals. FISH probes are **A** CD2-67 (chromosome 1), **B** CD2-68 (chromosome 2), **C** CD2-4 (chromosome 3), **D** CD2-43 (chromosome 4), **E** CD2-29 (chromosome 5) and **F** pYK205 (chromosome 1). **C**, **D** and **E** are pseudocolor images obtained by laser scanning confocal microscopy. *Arrows* indicate chromosomes showing FITC signals. Bars represents 2 μm

was derived from λ235, which maps at 43.9 of chromosome 1. This 11 kb sequence was detected on the middle of metacentric chromosomes (Fig. 4F).

Discussion

In the present study, we localized genomic DNA sequences greater than 11 kb in size on *Arabidopsis* chromosomes. This could only be reliably achieved using the floral tissues, in which more extended prometaphase chromosomes are frequently observed (Fig. 2). The discrepancy in efficiency of hybridization between root and floral chromosomal preparations might be caused by the difference in chromatin structure between the two tissues. Chromatin structure is known to be altered and sensitive to DNase I and other nucleases when the genes involved are transcribed actively (for review see Spiker 1985). There is no direct evidence that more diverse genes are transcribed actively in flower buds than in roots of *A. thaliana*. In tobacco, however, the flower petals were shown to contain about 6,000 diverse mRNAs that are not expressed in other organs such as roots (Kamalay and Goldberg 1980).

In a large number of plant species, various kinds of repetitive DNA sequences have been successfully mapped to mitotic metaphase chromosomes made from root-tips. In *Arabidopsis*, ribosomal RNA genes and 180 bp centromeric sequences could be localized with ease and high efficiency on the nucleolar organizing and centromeric regions, respectively (Murata et al. 1990, 1994; Maluszynska and Heslop-Harrison 1991), but the degree of staining with PI suggested that these regions are more condensed than other chromosomal regions (Murata et al. 1994). This suggests that the degree of chromatin condensation itself is not the only factor affecting the efficiency of hybridization.

Genomic clones harboring large inserts such as the cosmid clones used here are more likely to contain repetitive DNA sequences. This problem was encountered in the mapping of human chromosomes by FISH with cosmid clones, and therefore, an appropriate competitor such as non-labeled total human DNA should be added to the probe mix (e.g., Lichter et al. 1990). In the present study

with cosmid clones as probes, however, almost no hybridization signals from ubiquitous repetitive sequences were detected even without the addition of non-labeled genomic DNA of *A. thaliana*. This may be due to a low amount of repetitive sequences in the *Arabidopsis* genome, as well as the fact that the cosmid clones were prescreened to eliminate highly repetitive sequences (Nam et al. 1989). Furthermore, the chromosomal locations of the cloned sequences shown here by FISH were mostly correlated with the map positions determined by RFLP (restriction fragment length polymorphism) analyses. This highly efficient and specific detection without signal amplification suggests that the cosmid clones will be useful markers to identify *Arabidopsis* chromosomes.

Since flower petals or corolla tissues have also been used for making chromosome preparations in *Nicotiana* (Burns 1964) and *Solanum* (Kussel and Marks 1970), the present FISH technique using chromosome preparations from floral tissues is also applicable to other plant species. In plants, preparations of mitotic metaphase chromosomes have been made almost only from root meristematic tissues. This might have delayed progress in the chromosomal mapping of plant genes.

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References

Ambros PF, Matzke MA, Matzke AJM (1986) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 94:11–18

Arabidopsis Biological Resource Center (1994) Seeds and DNA stock list. Ohio State University, Columbus, Ohio

Burns JA (1964) A technique for making preparations of mitotic chromosome from Nicotiana flowers. Tobacco Sci 8:1–2

Chang C, Bowman JL, DeJohn AW, Lander ES, Meyerowitz EM (1988) Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. Proc Natl Acad Sci USA 85:6856–6860

Gustafson JP, Butler E, McIntyre CL (1990) Physical mapping of a low-copy DNA sequence in rye (*Secale cereale* L.). Proc Natl Acad Sci USA 87:1899–1902

Huang PL, Hahlbrock K, Somssich IE (1988) Detection of a single-copy gene on plant chromosomes by in situ hybridization. Mol Gen Genet 1211:143–147

Johnson GD, Araujo GM (1981) A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 43:349–350

Kamalay JC, Goldberg RB (1980) Regulation of structural gene expression in tobacco. Cell 19:935–946

Kussel R, Marks GE (1970) Making chromosome counts in solanum using corolla tissue. Potato Res 13:151–153

Lichter P, Tang CJC, Call K, Hermanson G, Evans GA, Housman D, Ward DC (1990) High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science 247:64–69

Maluszynska J, Heslop-Harrison JS (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J 1:159–166

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Mouras A, Saul MW, Essad S, Potrykus I (1987) Localization by in situ hybridization of a low copy chimaeric resistance gene introduced into plants by direct gene transfer. Mol Gen Genet 207:204–209

Murata M, Varga F, Maluszynska J, Grundler P, Schweizer D (1990) Chromosomal localization of the ribosomal RNA genes in *Arabidopsis thaliana* by in situ hybridization. Abstracts of 4th International Conference on Arabidopsis Research, Vienna, p 5

Murata M, Nakata N, Yasumuro Y (1992) Origin and molecular structure of a midget chromosome in a common wheat carrying rye cytoplasm. Chromosoma 102:27–31

Murata M, Ogura Y, Motoyoshi F (1994) Centromeric repetitive sequences in *Arabidopsis thaliana*. Jpn J Genet 69:361–370

Nam HG, Giradat J, den Boer B, Moonan F, Loos WDB, Hauge BM, Goodman HM (1989) Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. Plant Cell 1:699–705

Shen D, Wang Z, Wu M (1987) Gene mapping on maize pachytene chromosomes by in situ hybridization. Chromosoma 95:311–314

Spiker S (1985) Plant chromatin structure. Annu Rev Plant Physiol 36:235–253

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Genetics

Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial artificial chromosomes

(physical mapping/genomic DNA clones)

JIMING JIANG*†, BIKRAM S. GILL*§, GUO-LIANG WANG‡, PAMELA C. RONALD‡, AND DAVID C. WARD†

*Department of Plant Pathology, Kansas State University, Manhattan, KS 66506-5502; †Department of Genetics, Yale University School of Medicine, New Haven, CT 06510-8005; and ‡Department of Plant Pathology, University of California, Davis, CA 95616

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ABSTRACT **Fluorescence *in situ* hybridization (FISH) is a powerful tool for physical mapping in human and other mammalian species. However, application of the FISH technique has been limited in plant species, especially for mapping single- or low-copy DNA sequences, due to inconsistent signal production in plant chromosome preparations. Here we demonstrate that bacterial artificial chromosome (BAC) clones can be mapped readily on rice (*Oryza sativa* L.) chromosomes by FISH. Repetitive DNA sequences in BAC clones can be suppressed efficiently by using rice genomic DNA as a competitor in the hybridization mixture. BAC clones as small as 40 kb were successfully mapped. To demonstrate the application of the FISH technique in physical mapping of plant genomes, both anonymous BAC clones and clones closely linked to a rice bacterial blight-resistance locus, *Xa21*, were chosen for analysis. The physical location of *Xa21* and the relationships among the linked clones were established, thus demonstrating the utility of FISH in plant genome analysis.**

Fluorescence *in situ* hybridization (FISH) has become an important *in situ* hybridization tool because of its amenability of coupling with highly sensitive charge coupled device (CCD) camera and digital imaging analysis and its potential for mapping of several probes simultaneously. In humans, DNA probes smaller than 1 kb have been mapped on metaphase chromosomes by FISH (1) and DNA fragments separated by as little as 50–100 kb have been ordered within interphase nuclei or on prophase chromosomes (2–4). Ried *et al.* (5) mapped seven probes simultaneously by using a combinatorial fluorescence and digital imaging analysis. The success of FISH analysis in humans has also been facilitated by the application of hybridization techniques that suppress cross-hybridization from repetitive DNA sequences in the genomic clones by preannealing with unlabeled human genomic DNA in the hybridization mixture. Thus, specific clones can be isolated from cosmid, P1, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC) libraries and used directly for FISH mapping. Large numbers of genomic clones from chromosome-specific libraries have been used successfully to generate high-density cytological maps (6–8).

In plants, *in situ* hybridization techniques have been used mainly for mapping repetitive DNA sequences and multicopy gene families (9). Mapping of low or single-copy sequences has proven difficult compared to that in humans. In most of the successful *in situ* hybridization experiments in plants, the target DNA sequences on chromosomes were >10 kb long (10–14). Mapping of small probes was also reported (15); however, the frequency of signal detection was very low and the technique is not very reliable and reproducible (9). The relatively low sensitivity of *in situ* hybridization (including FISH) in plants is arguably due to the presence of rigid cell walls and cytoplasmic debris and the more pronounced condensation of plant metaphase chromosomes.

Because of the difficulty of using small single-copy sequence probes, genomic DNA cloned in large insert vectors should be a viable alternative approach to map genetic loci in plants. Even if repetitive sequences account for 90% of plant genomic DNA, a 100-kb genomic clone theoretically should contain ≈10 kb of unique sequence, enough to generate a good signal in most plant species examined to date. In the present work, we demonstrate the application of BAC clones for FISH mapping in rice (*Oryza sativa* L.).

MATERIALS AND METHODS

Rice BAC Clones. A rice BAC library was constructed from the rice line IR-BB21 containing *Xa21*, a bacterial blight disease-resistance locus derived from a wild African species, *Oryza longistaminata* (16). Five BAC clones linked to *Xa21* and five random clones (Table 1) from this library were used for FISH mapping. Five additional random clones from a different BAC library were kindly provided by R. A. Wing (Texas A&M University) (Table 1). The insert sizes of the mapped BAC clones range from 40 to 220 kb.

Strains. Rice lines DV85 and IR-BB21 were used in FISH mapping. DV85 is an indica rice cultivar. Line IR-BB21 is a nearly isogenic line (NIL) for *Xa21* that was constructed by backcrossing *O. longistaminata* (*Xa21* donor parent) five times to the recurrent parent IR24 followed by five selfings (18).

Chromosome Preparation. Seeds were germinated on moist filter paper in Petri dishes. Roots 1–2 cm long were cut and directly fixed in methanol/glacial acetic acid (3:1) for several hours up to a few days. The fixed root tips were washed thoroughly with 0.01 M citrate buffer (sodium citrate/citric acid, pH 4.8) and digested in 6% (wt/vol) cellulase (Calbiochem) and 2% (wt/vol) pectolyase (Sigma) for 30 min at 37°C. The enzymes were carefully washed from the softened material and replaced with 0.01 M citrate buffer for 10 min; the root tips were subjected to a hypotonic treatment in distilled water for 20 min and transferred to ethanol-washed glass slides. The tissue was macerated in a drop of methanol/glacial acetic acid (1:1) with a razor blade. The slides were air dried and stored in a −80°C freezer. The slides were treated with an ethanol series (70%, 90%, and 100% ethanol; 5 min each) just prior to *in situ* hybridization.

Probe Labeling. BAC DNA was isolated by an alkaline lysate method (19). The purified DNA was labeled by standard nick-translation reaction mixtures containing biotin (bio)-11-

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Abbreviations: bio, biotin; dig, digoxigenin; FISH, fluorescence *in situ* hybridization; CCD, charge coupled device; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; RFLP, restriction fragment length polymorphism; YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome; PFGE, pulsed-field gel electrophoresis; NIL, nearly isogenic line.

§To whom reprint requests should be addressed.

Table 1. BAC clones used in the present study

Name	Insert size, kb	Description*	Interphase signals	Repetitive sequences†	Source
UCD103-1	100	RG103	2	ND	UC–Davis
UCD103-5	90	RG103	2	ND	UC–Davis
UCD103-7	120	RG103	2	ND	UC–Davis
UCD818-13	85	pTA818	2	ND	UC–Davis
UCD818-14	40	pTA818	2‡	ND	UC–Davis
UCD2	220	Random	2	−	UC–Davis
UCD6	140	Random	2	−	UC–Davis
UCD11	130	Random	2	−	UC–Davis
UCD16	140	Random	2	+	UC–Davis
UCD18	170	Random	2	+	UC–Davis
TQ7A1	100	Random	2	−	Texas A&M
TQ7A3	140	Random	2	−	Texas A&M
TQ7A4	135	Random	2 + 2 minor	+	Texas A&M
TQ7A5	130	Random	2	−	Texas A&M
TQ7A8	110	Random	2	−	Texas A&M

*Random clones or clones isolated using DNA markers RG103 and pTA818 (16, 17).
†−, Discrete signals were observed without competitive genomic DNA; +, discrete signals were observed only with competitive genomic DNA; ND, no data.
‡Two spots were observed in normal rice line DV85, while four spots were observed in IR-BB21.

dUTP or digoxigenin (dig)-11-dUTP, and labeled probes were purified by passage through Sephadex G-50 columns. Although most BAC DNA preparations contained some bacterial genomic DNA, this contaminating DNA does not interfere with the FISH analysis, indicating that *Escherichia coli* does not share enough homologous DNA with rice to give detectable FISH signals. For two-color FISH, two different probes were labeled with bio-11-dUTP and dig-11-dUTP, respectively, while in three-color FISH the third probe was labeled combinatorially with 50% bio-11-dUTP and 50% dig-11-dUTP.

***In Situ* Hybridization and Detection.** The *in situ* hybridization protocol was modified slightly from that of Rayburn and Gill (20). About 10 ng of labeled BAC DNA was used for each slide in a hybridization mixture with 50% formamide/10% dextran sulfate/2× SSC/10 μg of salmon sperm DNA/≈1 μg of Cot-1 fraction of rice genomic DNA. The mixture was denatured at 80°C for 10 min, centrifuged briefly, and preannealed at 37°C for 1 hr before applying to slides. Slide-bound chromosomal DNA was denatured in a solution of 70% formamide in 2× SSC for 1.5 min at 80°C and dehydrated in a −20°C ethanol series (70%, 90%, and 100% ethanol; 5 min each). Ten microliters of hybridization mixture was applied to each slide and sealed under a coverslip (18 × 18 mm) with rubber cement.

After overnight incubation at 37°C, the coverslips were removed and the slides were washed at room temperature in 2× SSC for 5 min, at 45°C in 2× SSC for 10 min, at room temperature in 2× SSC for 5 min, and at room temperature in 1× PBS (phosphate-buffered saline) for 5 min. The biotinylated probes were detected with fluorescein isothiocyanate-conjugated avidin (FITC–avidin) (Vector Laboratories) and the dig-labeled probes were detected with a rhodamine-conjugated anti-dig antibody (Boehringer Mannheim). 4′,6-Diamidino-2-phenylindole (DAPI) was used as a chromosome counterstain in multicolor FISH while propidium iodide (PI) was used as a chromosome counterstain in single-color FISH with biotinylated probes.

Digital Imaging. Images were obtained with a Zeiss Axioskop epifluorescence microscope coupled to a cooled CCD camera (PM512; Photometrics; Tucson, AZ). Camera control and digital image acquisition (8-bit gray scale) were implemented with an Apple Macintosh IIx computer. Fluorophores were selectively imaged with filter cubes especially prepared by Zeiss (filter 487910 for fluorescein, filter 487915 for rhodamine and PI, and filter 487901 for DAPI) to minimize image registration problems and merged as described (5). It is worth emphasizing that although a CCD imaging system was used, the signals of the probes were clearly visible by eye through the microscope.

RESULTS

Metaphase and Interphase Mapping of BAC Clones. To investigate whether the presence of the repetitive sequences in the BAC clones would generate high levels of nonspecific hybridization, FISH was performed on 10 random BAC clones in the absence of Cot-1 rice genomic DNA. Surprisingly, discrete chromosomal loci could be identified for 7 of the 10 clones. Although minor hybridization signals were distributed uniformly over all the chromosomes and nuclei, the stronger signals from the unique sequences of the BAC clones were clearly distinguishable from the background (Fig. 1*a*). However, the signal/noise contrast with these BACs was greatly improved in the presence of competitive rice genomic DNA. Distinct FISH signals were observed with the other three BAC clones only in the presence of the rice competitor DNA (compare Fig. 1 *b* and *c*). FISH signals were obtained from all the BACs analyzed, including clone 818-14 that contained a relatively small insert of ≈40 kb (Table 1).

Both metaphase chromosomes and interphase chromatin were scored in this FISH analysis. Clear signals were observed in 50–90% of the interphase nuclei and in >90% of the complete metaphase spreads. In most cases, signals were observed on both chromatids on each pair of homologous metaphase chromosomes, although merged signals from two sister chromatids were also observed frequently. As expected, prometaphase chromosomes generally gave better resolution (relative position on a specific chromosome arm) of the signals than the more condensed metaphase chromosome preparations.

Fourteen of the 15 BAC clones analyzed in line DV85 gave two FISH signals in interphase nuclei. This suggests that the inserts in these clones constitute a single piece of rice genomic DNA and thus are not chimeric. The remaining clone, TQ7A4, gave in addition to two major spots one or two minor spots in some of the interphase nuclei. We do not know whether these minor spots reflect a chimeric DNA insert or the presence of related sequences at a second locus in the genome.

We next evaluated the utility of multicolor FISH for mapping several probes simultaneously. Rice prometaphase chromosomes have different DAPI or PI staining intensities in different parts of the chromosomes due to uneven condensation. In general, for most of the chromosomes, there is a decreased staining toward the telomeres. This staining pattern

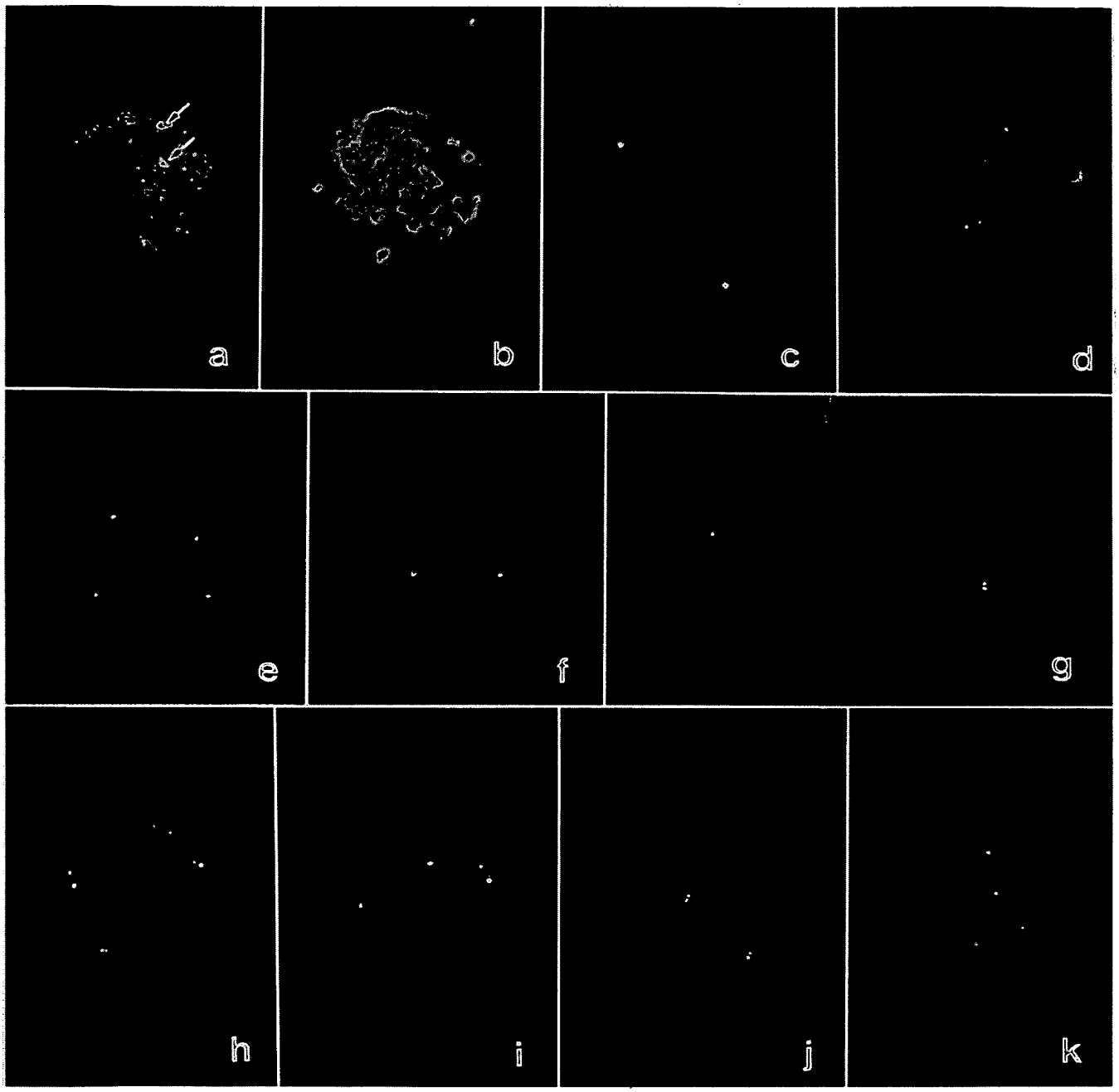

FIG. 1. (*a*) Interphase mapping of BAC clone TQ7A5. Cot-1 rice genomic DNA was not applied during hybridization. Two major spots (arrows) can be observed along with the minor spots all over the nucleus. The cell was not counterstained. (*b*) Interphase mapping of BAC clone UCD18. Cot-1 genomic rice DNA was not applied. No discrete signals can be observed. The cell was not counterstained. (*c*) Interphase mapping of UCD18. Cot-1 rice genomic DNA was applied during hybridization. Two discrete signals can be observed. The cell was counterstained by PI. (*d*) Cohybridization of BAC clone TQ7A1 (labeled with bio-11-dUTP and detected with FITC–avidin; yellow spots) with telomeric sequence probe pAtT4 (labeled with dig-11-dUTP and detected with rhodamine-conjugated anti-dig antibody; red spots). TQ7A1 is located on the proximal region of the long arm of a pair of unidentified chromosomes. (*e*) Interphase three-color FISH mapping of three random BAC clones: TQ7A5 (yellow), UCD2 (red), and UCD6 (white). (*f*) Interphase mapping of 103-1 (yellow) and 103-7 (red) in IR-BB21. Signals from the two clones overlap. (*g*) Metaphase mapping of the bacterial blight-resistance locus *Xa21* in IR-BB21 using a closely linked BAC clone 103-7. Signals are located at the middle of the long arm of chromosome 11. (*h*) Metaphase mapping of 818-14 in IR-BB21. Signals are located on two pairs of chromosomes. (*i*) Interphase mapping of three BAC clones in IR-BB21. Clone 818-13, labeled by bio-11-dUTP and detected by FITC, two relatively small yellow spots; clone 103-7, labeled by bio-11-dUTP and detected by FITC, two relatively large yellow spots; clone 818-14, labeled by dig-11-dUTP and detected by rhodamine, two small and two large red spots. Note: two small yellow spots overlap with two small red spots; two large yellow spots overlap with two large red spots. (*j*) Metaphase mapping of 818-13 (yellow) and 818-14 (red) in DV85. Signals from the two clones overlap. Signals are located at a proximal region of a pair of unidentified chromosomes. (*k*) Interphase mapping of three BAC clones in DV85: 818-13 (yellow), 103-7 (yellow), and 818-14 (red). Signals from 818-13 and 818-14 overlap. Signals from 103-7 are independent from those of 818-13 and 818-14.

makes it difficult to locate the true ends of the chromosomes and the relative chromosomal location of the probes. To address this problem, we cohybridized a telomeric sequence probe, pAtT4 derived from *Arabidopsis thaliana* (21), with specific BAC clones. Fig. 1*d* shows the cohybridization of BAC clone TQ7A1 with pAtT4. TQ7A1 is located on the proximal region of the long arm of a pair of unidentified chromosomes. Hybridization signals from pAtT4 are visible on both ends of this pair of chromosomes.

Fig. 1*e* demonstrates the interphase mapping of three different BAC probes by three-color FISH. The signals from the third probe were pseudocolored with white to distinguish it from the other two probes. Multicolor FISH was also used to analyze the relative position of physically closely related BAC clones (see below).

Physical Mapping of BAC Clones Linked to *Xa21*. A rice bacterial blight disease-resistance locus, *Xa21*, was transferred from a wild species, *O. longistaminata*, into cultivated rice (18, 22). Several closely linked DNA markers to *Xa21* were identified in NIL IR-BB21 (17). BAC clones were isolated by using these DNA markers as probes (16) and analyzed by FISH.

Three BAC clones were identified by the eight-copy restriction fragment length polymorphism (RFLP) marker, RG103, that cosegregates with *Xa21*. The three clones can be divided into two different nonoverlapping groups according to their Southern hybridization patterns: group 1 includes BAC 103-1 and BAC 103-5; group 2 includes BAC 103-7 (P.C.R., unpublished results). Most copies of RG103 hybridize to a 230-kb *Sfi* I DNA fragment by pulsed-field gel electrophoresis (PFGE) analysis (P.C.R., unpublished results). FISH analysis showed that all three clones hybridized to the same chromosomal region. Two-color FISH with BACs 103-1 and 103-7 confirmed their close linkage as their hybridization signals overlapped on both metaphase chromosomes and interphase nuclei (Fig. 1*f*). This result is in good agreement with PFGE analysis. The three BAC clones were physically mapped at the middle of the long arm of chromosome 11 (Fig. 1*g*) with a FL value of 0.50 (FL, fractional length, a fraction from the FISH signal to centromere over the total chromosome arm length). The chromosome identification is deduced from the position of RFLP marker RG103 in genetic linkage map 11 (23). The arm ratio (long arm/short arm) of chromosome 11 is 1.41 at somatic metaphase in NIL IR-BB21. However, chromosome 11 was identified as a metacentric chromosome by pachytene analysis in variety IR36 (24).

Two additional BAC clones, 818-13 and 818-14, were identified by DNA marker pTA818, originally derived from a random amplified polymorphic DNA band (RAPD818 in ref. 17). Clone 818-13 contains one copy of pTA818 that is monomorphic between the resistant and susceptible NILs. Clone 818-14 contains another copy of pTA818 that is polymorphic between the NILs and mapped to the *Xa21* locus on chromosome 11 (17). FISH analysis in NIL IR-BB21 showed that 818-13 has only one location. However, 818-14 has two different locations with one FISH site relatively small and the other relatively large in interphase nuclei. The two copies of 818-14 are clearly located on two different chromosomes by metaphase mapping (Fig. 1*h*). Two-color FISH showed that FISH signals from 818-13 overlapped with the smaller site of 818-14 and the larger site of 818-14 overlapped with clone 103-7 (Fig. 1*i*). Thus, 818-13 and the smaller copy of 818-14 are located on a different chromosome from 103-7 and are not linked to *Xa21*. In the regular rice line DV85, 818-14 has only one location and overlaps with the signals from 818-13 on both interphase and metaphase chromosomes (Fig. 1*j*). Cohybridization of probes 818-13, 818-14, and 103-7 indicates that clones 818-13 and 818-14 are located on a different chromosome from clone 103-7 in DV85 (Fig. 1*k*). Both 818-13 and 818-14 are located in a proximal region of a pair of unidentified chromosomes (Fig. 1*j*).

DISCUSSION

We have demonstrated that large genomic clones can be used for *in situ* hybridization mapping in rice, even though they may contain highly repeated DNA sequences. All BAC clones analyzed in the present study generated strong FISH signals, even with inserts as small as 40 kb. In a similar fashion, we have shown that cosmid clones can also be applied to FISH mapping of the rice genome (unpublished results). This technique provides an excellent approach to overcome the difficulty of *in situ* hybridization mapping using single- or low-copy sequences in plants. The successful application of this technique in other plant species will depend on both the size of the genomic clones analyzed and the percentage of repetitive DNA sequences in the genome. The rice genome contains a large percentage (≈75%) of single- or low-copy number DNA (25, 26) and this may contribute to the successful application of small genomic clones in FISH analysis. For other plant species, such as wheat (*Triticum aestivum* L.), with genomes containing >80% repetitive DNA sequences (27), relatively large clones will be needed to ensure that enough unique sequence will be available to generate distinctive signals.

FISH analysis provides a rapid method to determine the chimeric status of a genomic clone. A high percentage of chimeric clones in a genomic library, such as that observed in many YAC libraries, complicates map construction considerably. Thus, it is important to know the general status of chimerism in a library. Although chimerism can be analyzed by genetic analysis of the ends of the DNA insert in a specific clone, this approach is time consuming compared to data obtained by FISH. This is of particular importance in plant species with a high percentage of repetitive sequences, since most of the insert ends contain repetitive sequences that cannot be used for RFLP mapping or generating PCR probes for hybridization screening (28). The disadvantage of FISH analysis is that the FISH signals neither distinguish duplicated sequences from true chimerism nor discriminate between chimerism and cocloning or mixed colony propagation events. Nevertheless, comparative studies of FISH and molecular methods for determining chimerism in human YAC clones have demonstrated a high level of concordance (29). It is interesting to note that all clones from the rice BAC library derived from IR-BB21 (16) hybridized to a single location; similar results were obtained with clones isolated from a sorghum BAC library (28). This indicates that these libraries have a very low level of chimerism as compared to a rice YAC library in which 40% of the clones are chimeric (30).

Rice chromosomes are small and very difficult to identify individually on the basis of morphological differences. Reproducible banding techniques are not available for identification of the rice chromosomes. Only investigators with considerable experience and skill are able to identify different chromosomes by using well spread somatic prometaphase or meiotic pachytene preparations (24, 31). FISH analysis using genomic clones that contain genetically linked markers should provide a new technique for rice chromosome identification. BAC clones specific to each chromosome can be isolated from libraries and used as chromosome-specific FISH markers. It was shown previously that combinatorial labeling of probes with three different modified nucleotides permitted the detection of seven different probes simultaneously (5). Further development in this field should make it possible to visualize even more probes simultaneously (32). Therefore, it is possible in the future that all 12 pairs of rice chromosomes could be identified in a single multicolor FISH experiment with chromosome-specific FISH markers.

Chromosome-specific FISH markers will be valuable for aneuploid identification, such as trisomics, by interphase mapping. Well-spread metaphase or prometaphase cells, usually difficult to obtain, are not necessary in interphase mapping.

Chromosome-specific FISH markers also can be used to analyze other genetic stocks, such as translocation stocks. A series of markers located at different regions of a chromosome can be established and used to analyze specific translocation stocks, so that the breakpoint can be localized between two specific markers. Another possible application of chromosome-specific FISH markers is to analyze the structure of chromosomes from related species. For example, the gene synteny and homologous relationship of chromosomes from a wild rice species to a particular rice linkage group can be quickly analyzed by FISH mapping of these markers.

FISH mapping of the *Xa21*-related genomic clones demonstrated that this technique provides a valuable supplemental tool for map-based cloning in plants. This technique can be used for rapid determination of the chromosome location of a group of genomic clones. Chimeric clones or clones located on different chromosomes can be eliminated from the walking analysis. Characterization of the relationship among 818-13, 818-14, and 103-7 clones provided important information that would be difficult to obtain by conventional genetic and PFGE analysis. Based on the previous genetic data, the pTA818 sequence located in the 818-13 clone was monomorphic between the recurrent parent and NIL IR-BB21 and could not be mapped in the progeny (17). In addition, initial PFGE results from NIL IR-BB21 indicated that all DNA sequences in 818-13, 818-14, and 103-7 were located in the same chromosomal region (17). However, FISH analysis of these three clones quickly and clearly established that 818-14 is located very close to 103-7 but 818-13 is located on a different chromosome in NIL IR-BB21. Our recent PFGE data confirmed this result (P.C.R., unpublished data). The close genetic and physical linkage between 818-14 and 103-7 indicates that these clones provide a good starting point for chromosomal walking to the *Xa21* disease-resistance locus.

Interestingly, in both NIL IR-BB21 and DV85, a FISH signal from the 818-14 probe overlaps with the 818-13 signal. This result is likely due to the homology of a 1-kb pTA818 sequence shared by 818-13 and 818-14. It is not known how far the homology extends. In DV85, 818-14 maps to a single location and is therefore unlikely to be a chimeric clone. The two locations of 818-14 in NIL IR-BB21 could be explained as follows: in *O. longistaminata*, the pTA818 sequence may have transposed from an unlinked chromosome to the *Xa21* locus on chromosome 11, resulting in the observed sequence duplication in NIL IR-BB21. In support of this speculation, we recently discovered a high degree of similarity of the pTA818 sequence to a portion of the *Antirrhinum* gene *Tam1*, a member of the CACTA family of transposable elements (P.C.R., unpublished data).

An important future application of FISH mapping in plants is to determine the physical distance between genetically mapped clones. In a map-based cloning strategy, one of the most important steps is to estimate the physical distance separating two tightly linked markers flanking the target gene. Because of the uneven distribution of recombination along the physical length of plant chromosomes (33, 34), it could be difficult to determine such physical distance by conventional PFGE analysis. In these situations, FISH mapping can be applied to estimate the relative physical distance based on the separation distance of FISH spots in the interphase nuclei as demonstrated in humans (3). However, the relationship between separation distances of FISH signals in interphase nuclei and linear DNA distance in plant species remains to be established.

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1. Viegas-Péquignot, E., Berrard, S., Brice, A., Apiou, F. & Mallet, J. (1991) *Genomics* **9**, 210–212.
2. Lawrence, J. B., Villnace, C. A. & Singer, R. H. (1988) *Cell* **52**, 51–61.
3. Trask, B. J., Pinkel, D. & van den Engh, G. (1989) *Genomics* **5**, 710–717.
4. Inazawa, J., Ariyama, T., Tokino, T., Tanigami, A., Nakamura, Y. & Abe, T. (1994) *Cytogenet. Cell Genet.* **65**, 130–135.
5. Ried, T., Baldini, A., Rand, T. C. & Ward, D. C. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1388–1392.
6. Lichter, P., Tang, C. C., Call, K., Hermanson, G., Evans, G. A., Housman, D. & Ward, D. C. (1990) *Science* **247**, 64–69.
7. Kunz, J., Scherer, S. W., Klawitz, I., Soder, S., Du, Y.-Z., Speich, N., Kalff-Suske, M., Heng, H. H. Q., Tsui, L.-C. & Grzeschik, K.-H. (1994) *Genomics* **22**, 439–448.
8. Zheng, C.-J., Ma, N. S.-F., Dorman, T. E., Wang, M.-T., Braunschweiger, K., Soares, L., Schuster, M. K., Rothschild, C. B., Bowden, D. W., Torrey, D., Keith, T. P., Moir, D. M. & Mao, J.-I. (1994) *Genomics* **22**, 55–67.
9. Jiang, J. & Gill, B. S. (1994) *Genome* **37**, 717–725.
10. Ambros, P. F., Matzke, M. A. & Matzke, A. J. M. (1986) *Chromosoma* **94**, 11–18.
11. Simpson, P. R., Newman, M.-A. & Davies, D. R. (1988) *Chromosoma* **96**, 454–458.
12. Schaff, D. A., Koehler, S. M., Mattews, B. F. & Bauchan, G. R. (1990) *J. Hered.* **81**, 480–483.
13. Lehfer, H., Busch, W., Martin, R. & Herrmann, R. G. (1993) *Chromosoma* **102**, 428–432.
14. Leitch, I. J. & Heslop-Harrison, J. S. (1993) *Genome* **36**, 517–523.
15. Gustafson, J. P. & Dillé, J. E. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8646–8650.
16. Wang, G. L., Holsten, T. E., Wang, H.-P. & Ronald, P. C. (1995) *Plant J.*, in press.
17. Ronald, P. C., Albano, B., Tabien, R., Abenes, L., Wu, K.-S., McCouch, S. & Tanksley, S. D. (1992) *Mol. Gen. Genet.* **236**, 113–120.
18. Khush, G. S., Bacalangco, E. & Ogawa, T. (1991) *Rice Genet. Newsl.* **7**, 121–122.
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
20. Rayburn, A. L. & Gill, B. S. (1985) *J. Hered.* **76**, 78–81.
21. Richards, E. J. & Ausubel, F. M. (1988) *Cell* **53**, 127–136.
22. Khush, G. S., Mackill, D. J. & Sidhu, G. S. (1989) *Proceedings of the International Workshop on Bacterial Blight of Rice* (Int. Rice Res. Inst., Los Banos, Philippines), pp. 207–217.
23. Causse, M., Fulton, T. M., Cho, Y. G., Ahn, S. N., Chunwonges, J., Wu, K., Xiao, J., Yu, Z., Ronald, P. C., Harrington, S. B., Second, G. A., McCouch, S. R. & Tanksley, S. D. (1994) *Genetics* **138**, 1251–1274.
24. Khush, G. S., Singh, R. J., Sur, S. C. & Librojo, A. L. (1984) *Genetics* **107**, 141–163.
25. Deshpande, V. G. & Ranjekar, P. K. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1223–1233.
26. McCouch, S. R., Kochert, G., Yu, Z. H., Wang, Z. Y., Khush, G. S., Coffman, W. R. & Tanksley, S. D. (1988) *Theor. Appl. Genet.* **76**, 815–829.
27. Smith, D. B. & Flavell, R. B. (1975) *Chromosoma* **50**, 223–242.
28. Woo, S.-S., Jiang, J., Gill, B. S., Paterson, A. H. & Wing, R. A. (1994) *Nucleic Acids Res.* **22**, 4922–4931.
29. Haldi, M., Perrot, V., Saumier, M., Desai, T., Cohen, D., Cherif, D., Ward, D. C. & Lander, E. S. (1994) *Genomics* **24**, 478–484.
30. Umehara, Y., Inagaki, A., Tanoue, H., Yasukochi, T., Nagamura, Y., Saji, S., Otsuki, Y., Fujimura, T., Kurata, N. & Minobe, Y. (1994) *Plant Breed.* **1**, 79–89.
31. Fukui, K. & Iijima, K. (1991) *Theor. Appl. Genet.* **81**, 589–596.
32. Ballard, S. G. & Ward, D. C. (1993) *J. Histochem. Cytochem.* **41**, 1755–1759.
33. Curtis, C. A. & Lukaszewski, A. J. (1991) *Theor. Appl. Genet.* **81**, 245–252.
34. Werner, J. E., Endo, T. R. & Gill, B. S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11307–11311.

Theor Appl Genet (1994) 87:893–899

Variability in rDNA loci in the genus *Oryza* detected through fluorescence in situ hybridization

K. Fukui[1], N. Ohmido[1], G. S. Khush[2]

[1] Hokuriku National Agricultural Experiment Station, Joetsu 943-01, Japan
[2] International Rice Research Institute, Manila, P.O. Box 933, The Philippines

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Abstract. The 17*s*–5.8*s*–25*s* ribosomal RNA gene (rDNA) loci in *Oryza* spp. were identified by the fluorescence in-situ hybridization (FISH) method. The rDNA loci were located on one-to-three chromosomes (two-to-six sites) within the eight diploid *Oryza* spp. One of the rDNA loci gave the weakest hybridization signal. This locus is reported for the first time in the genus *Oryza*. The chromosomes containing the rDNA loci were determined to be numbers 9, 10 and 11 in descending order of the copy number of rDNA. The application of image analysis methods, after slide preparation treatments (post-treatments), and the use of a thermal cycler, greatly improved the reproducibility of the results. The evolutionary significance of the variability of rDNA loci among the *Oryza* spp. is discussed.

Key words: Fluorescence in-situ hybridization (FISH) – Ribosomal DNA – Genus *Oryza* – Image analysis – NOR variability

Introduction

Rice chromosomes with a large 17s–5.8s–25s ribosomal RNA gene (rDNA) array have been identified as the satellite chromosomes by their characteristics (Fukui and Iijima 1991; Yanagisawa et al. 1991). They are also recognized as the chromosomes with nucleolar organizing regions (NOR chromosomes).

In cultivated rice, *Oryza sativa* L. ssp *japonica*, one pair of NOR chromosomes was reported by Kurata and Omura (1978) and Fukui and Iijima (1991). This NOR chromosome was designated as no. 11 (Fukui and Iijima 1991) but according to the new system for numbering rice chromosomes, has now been redesignated as no. 9 (Khush and Kinoshita 1991; Fukui and Iijima 1992). By contrast, two pairs of NOR chromosomes were reported in *O. sativa* ssp. *indica* (Wu et al. 1985).

Although the rDNA-containing chromosomes show conspicuous characteristics as satellite chromosomes, they are sometimes difficult to identify morphologically when the copy number of the rDNA units at the locus is small (Leitch and Heslop-Harrison 1992). The in-situ hybridization (ISH) method (Appels et al. 1980; Hutchinson and Miller 1982; Rayburn and Gill 1985) offers a way out of this impasse since it is based on the detection of rDNA loci directly by molecular hybridization. Using this technique one rDNA locus was identified on chromosome 9 in japonica rice. (Fukui et al. 1987; Fukui 1990; Iijima et al. 1991) while two rDNA loci were detected in indica rice (Islam-Faridi et al. 1990).

Although ISH is now widely employed in cytogenetic analysis, it is time consuming and strict experimental protocols are needed for its success. Therefore we have developed a reproducible and convenient fluorescence ISH (FISH) technique in conjunction with imaging methods, the use of a thermal cycler, and various post-treatments. As a result, clear fluorescent signals were reproducibly obtained and a new rDNA locus was detected in two diploid wild rice species.

Materials and methods

Plant materials and cytological procedures

Nine rice species, as listed in Table 1, were obtained either from the gene bank of the National Institute of Genetics (Mishima

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Correspondence to: K. Fukui

411, Japan) or Hokuriku National Agricultural Experiment Station (Joetsu 943-01, Japan). Seeds of trisomic lines for chromosomes 9 and 10, and their original variety, IR24, were supplied by Dr. Tsugufumi Ogawa (Kyushu National Agricultural Experiment Station, Chikugo 830, Japan). Seeds were germinated on moist filter paper in Petri dishes at 27 °C under continuous illumination. Root tips 1–2 cm long were excised and fixed in ethanol: acetic acid (1 : 1). They were stored at −20 °C for about 1 week before examination.

The procedures for sample preparation were according to the protocol described by Fukui and Iijima (1991, 1992) with minor modifications as follows: (1) the glass slides which were used for in-situ hybridization were coated with a 0.1% poly-L-lysine solution (Sigma); (2) enzymatic maceration was carried out in a 1.5-ml Eppendorf tube at 37 °C for 60–90 min; (3) the root tips were treated for 15–30 min in a decompression chamber before maceration and (4) the chromosome preparations were flame-dried.

Fluorescence in-situ hybridization (FISH)

Chromosome samples on a glass slide were subjected to four sequential 'post-treatments'. Firstly, they were treated with an enzymatic mixture (2% Cellulase Onozuka RS, Yakult Honsha, Co., Ltd., Tokyo, 1.5% Macerozyme R-200, Yakult Honsha, and 0.3% Pectolyase Y-23, Seishin Pharmaceutical Ltd., Tokyo, 1mM EDTA, pH 4.2) in 2 × SSC at 37 °C for 30 min. Secondly, they were treated with 1 mg/ml Proteinase K (Wako Pure Chemical Industries Ltd., Osaka) at 37 °C for 30 min. Thirdly, they were washed in 45% acetic acid for 5 min. They were completely dehydrated through a 70, 95 and 99% ethanol series for 10 min each and were air-dried. Finally, they were treated with 100 µg/ml RNase A (Sigma) in 2 × SSC at 37 °C for 60 min.

The rDNA probe (Sano and Sano 1990) was kindly supplied by Dr. Yoshio Sano, National Institute of Genetics, Mishima 411, Japan. This probe is 3.8 kb in length and covers most of the coding regions of the ribosomal RNA genes and the flanking spacer regions. The probe was labelled by a random primer labelling method with biothin-dUTP under the supplier's instructions. A 15-µl aliquot of the hybridization mixture containing 100 ng of biotinylated-rDNA in 50% formamide/2 × SSC was dropped on a glass slide. The solution was covered with a cover slip, sealed with liquid Arabian gum and then air-dried.

The glass slide with sealed cover slip was placed on a thermal cycler (PHC-3, Techne, Cambridge, UK) that had been remodelled by adding an 80 × 120 mm cast-aluminum flat plate. The programmed heating sequence was 70 °C for 6 min and 37 °C for 18 h.

The cover slips were removed and the slides were washed with 2 × SSC three times and once with 4 × SSC at 37 °C for 10 min each. A 70-µl aliquot of fluorescein isothiocyanate (FITC)-avidin conjugate (0.1 mg/ml, Boehringer Mannheim) was dropped onto the glass slides, which were then incubated at 37 °C for 60 min. After rinsing the FITC-avidin solution with BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) three times at 40 °C for 10 min each, a 70-µl biotinylated anti-avidin solution (1%, Vector Laboratory, Calif., USA.) was dropped onto the glass slides which were then incubated at 37 °C for 30 min. After brief washing with BT buffer, a 70-µl fluorescein-avidin solution (1%, Vector Lab.) was applied to each slide. The slides were again incubated at 37 °C for 30 min and then washed thoroughly with BT buffer three times at 40 °C for 10 min each.

Blocking was carried out three times before probe hybridization and before the immunological reaction with 5% bovine serum or goat serum albumin in BT buffer at 37 °C for 5 min.

The slides were counter-stained with a propidium iodide (PI) solution (50 µg/ml in phosphate buffer, pH 6.8, 12.5 mg/ml *p*-phenylendiamine dihydrochloride, with 90% glycerol) and were then examined by fluorescence microscopy.

Fluorescence microscopy and image analysis

A fluorescence microscope (Axiophot, Zeiss) with B- and G-light excitation filters (B10, G15) was used. A highly-sensitive color CCD camera (HCC-3600P, Floubel, Tokyo) was mounted on the microscope and the fluorescent images were directly frozen in the image frame memories of an image analysis system (VIDAS, Zeiss). All the B- and G-light excitation images were separately recorded in floppy disks and were subjected to image analysis.

Each image has 512 × 512 pixel matrix with 256 steps of a grey value for each pixel as in the images previously analyzed and reported by using the chromosome image analyzing system, CHIAS (Fukui 1985, 1986, 1988; Fukui et al. 1989). Necessary image manipulations consisting of shading correction, normalization, binalization, application of the median filter, and erasing of noise on the binary image, were carried out.

Details of each image filter and image manipulation were as reported previously (Fukui and Kakeda 1990; Fukui and Iijima 1991). Chromosomal areas and the signal regions were separately extracted from the respective G- and B-light images. For the fluorescent signals obtained in the B light, the original grey values were transformed to grey values ranging from 200 to 255. The grey values ranging from 0 to 199 were allocated to the pixels of the G-light image that demonstrated mainly chromosome images. The two grey images were combined into a single image.

Pseudocoloration using a look-up-table increased the definition of the image due to the differential coloration generated by computer imaging. The look-up-table was developed by trial and error by repeatedly comparing the original microscopic images with the computer-generated images (Fukui and Ito 1989; Fukui and Kamisugi 1991). The original source images both in B and G light were photographed using reversal color films (Fujichrome 100, ISO 100, Fuji Photo Film Co., Ltd., Tokyo). Digital images were photographed by a color image recorder (CIR-310 Nippon Avionics, Ltd., Tokyo) using reversal color films (Ektachrome 100, ISO 100, Kodak).

Results

Figure 1a shows the G-light excitation image of the chromosomes of *O. sativa*, ssp, *indica*, cv IR36. Two pairs of fluorescent signals were observed in B light (Fig. 1b). Figure 1c shows the intergrated image obtained by image manipulation. The current B or G excitation filter used in the experiment visualized either the yellowish-green fluorescence of FITC/fluorescein or the reddish fluorescence of PI. By image processing, the two fluorescent signals were integrated into a single image with yellowish signals on the reddish chromosomes. For basic information on the size and number of signals on the chromosomes, the visual recognition of the integrated image was markedly improved by image processing as shown in Fig. 1c. The four signal positions of IR36 were more precisely determined by using the integrated image compared with the two original images.

Fig. 1a–c. In-situ hybridization of IR36 chromosomes using the biotinylated rDNA probes. **a** Original photographic image of the chromosomes in G light. **b** Original photographic image of signals in B light. **c** Integrated image of both a and b images by imaging methods. The image was also digitally zoomed up. Bars indicate 5 μm for **a** and **b** and 3 μm for **c**

Figure 2 depicts eight representative examples of signal occurrence on the chromosomes in the nine *Oryza* species with six different genomes. The rDNA sites varied from one pair (Fig. 2a, g), two pairs (Fig. 2b, e, f), three pairs (Fig. 2c, d) and five pairs (Fig. 2h) within the chromosome complements. Although the size and intensity of the signals varied from sample to sample, a general pattern of signal size and intensity was evident. Two large and two medium-sized signals were most commonly observed in the

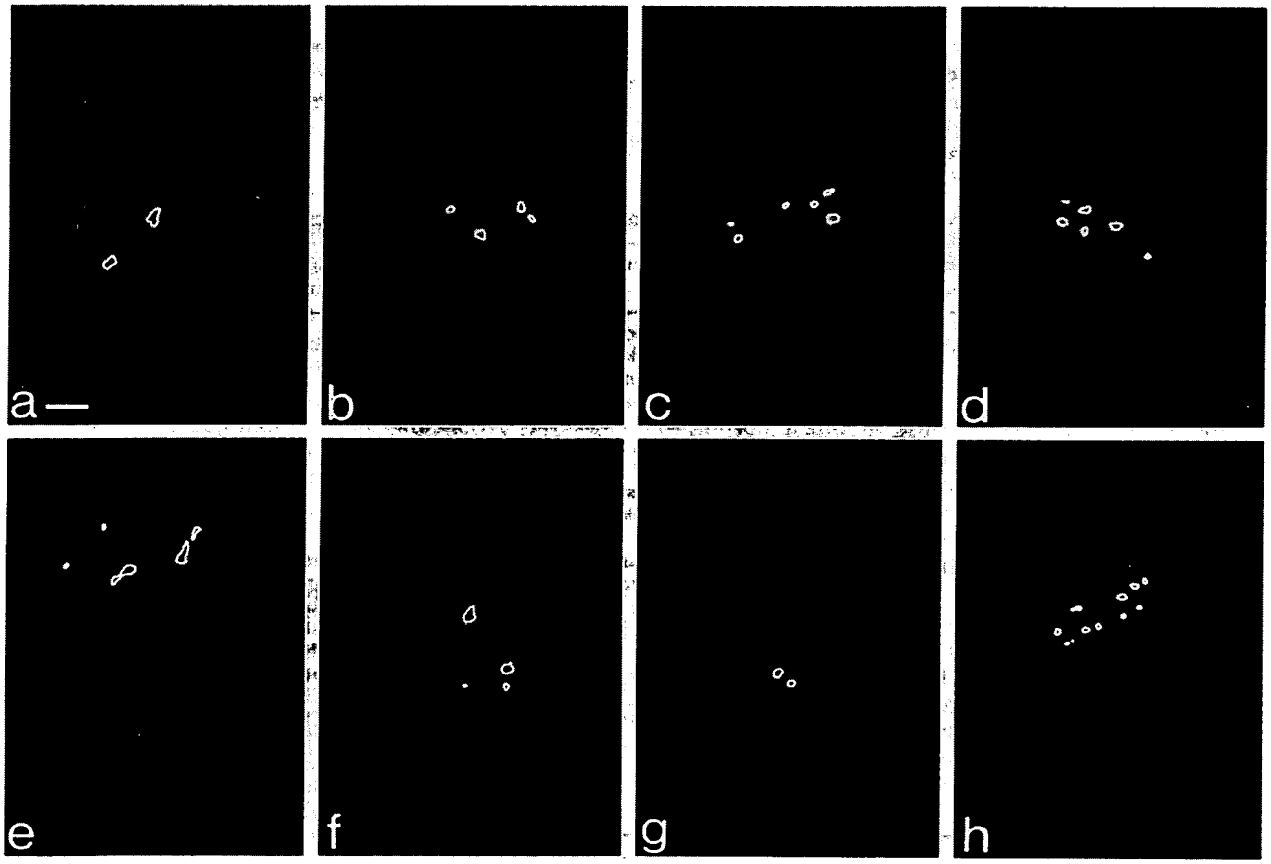

Fig. 2a–h. Fluorescent signals of rDNA sites in eight rice species. **a**, *O. sativa* ssp. *japonica*, CH79; **b**, *O. sativa* ssp. *javanica*, Inakupa; **c**, *O. punctata*; **d**, *O. officinalis*; **e**, *O. eichingeri*; **f**, *O. australiensis*; **g**, *O. brachyantha*; **h**, *O. latifolia*. Bar indicates 3 μm

species, giving the four signals as demonstrated in Fig. 2e and f. Two large-, two medium-, and two small-sized signals were observed in the species with six signals, although the differences between the large- and medium-sized signals were not always clear (Fig. 2c, d).

On occasions the secondary constriction, the adjoining part of the chromosome and the satellite were all fluorescent (Fig. 2e). Such signals were sometimes counted as two for one rDNA site, so that the signal number was sometimes three, four or even six (Fig. 2e). It was, however, possible to distinguish two genuine signals from the situation where two signals were created at one site by the chromosomal morphology observed in G excitation light. The secondary constrictions were also sometimes fluorescent (Fig. 2e).

Table 1 summarizes the number of rDNA loci detected by the FISH method in nine *Oryza* species with the A, A_{gp}, B, C, E, F and CD genomes. One or two rDNA loci were identified in the species with the A genome. One B genome species showed three rDNA loci. Two C genome species showed either two or three rDNA loci. Species with the E and F genomes exhibited either two or one rDNA loci, respectively. The number of rDNA loci in the D genome may be either two or three as in the case of the C genome species. Variability in the number of rDNA sites was thus observed between the species with different genomes, as well as between species with the same genome. Moreover the number of rDNA loci differed between the lines within a species.

Table 1 reports the results of a detailed examination of the number of rDNA loci amongst the A genome species.

Three japonica varieties, Nipponbare, Aikoku and Tushimaakamai, had one rDNA locus. Three indica varieties, Chinsurah Boro II, Kasalath and IR36, exhibited two rDNA loci. Two javanica rice varieties, Ketan Nanga from Indonesia and Inakupa from the Philippines, showed two rDNA loci. Three japonica varieties from China showed one rDNA locus. On the the other hand Kouketsumochi, a glutinous rice from southern China, showed two rDNA loci.

O. rufipogon, an A genome wild species, consists of two groups, a perennial type and an annual type (Oka 1983). One annual and two perennial types were examined. One or two rDNA loci were observed in the perennial. Two rDNA loci were detected in the annual.

Figure 3 shows the identification of the rDNA chromosomes by the FISH method using two trisomic lines and the parental variety, IR24. Two large- and two small-sized signals were observed on the chromosomes of disomic IR24 (Fig. 3a). Five signals were however discernible in both trisomic lines. All the fluorescent

Table 1. Cultivated rice (*O. sativa*) and the wild species studied and the number of rDNA loci detected

Species	Genome	Varietal group	Variety name	Source[a] and origin	Number of rDNA loci
O. sativa	AA	*japonica*	Nipponbare	HNAES, Japan	1
			Aikoku	HNAES, Japan	1
			Tushimaakamai	HNAES, Japan	1
			Tarizaohsien	HNAES, China	1
			Kouketsumochi	HNAES, China	2
			Ch78	NIG, China	1
			Ch79	NIG, China	1
		indica	Chinsurah Boro II	HNAES, India	2
			Kasalath	HNAES, India	2
			IR36	HNAES, India	2
		javanica	Ketan Nanga	HNAES, Indonesia	2
			Inakupa	NIG(221), Philippines	2
O. rufipogon	AA	Annual type		NIG(W0106), India	2
		Perennial type		NIG(W0149), India	2
				NIG(W1944),China	1
O. glumaepatula	A_{gp} A_{gp}			NIG(W1192), Brazil	1
O. punctata	BB			NIG(W1582), Chad	3
O. officinalis	CC			NIG(W0002), Thailand	3
O. eichingeri	CC			NIG(W1521), Uganda	2
O. australiensis	EE			NIG(W1538), Australia	2
O. brachyantha	FF			NIG(W1401), Sierra Leone	1
O. latifolia	CCDD			NIG(W0019), Unknown	5

[a] HNAES = Hokuriku National Agricultural Experiment Station
NIG = National Institute of Genetics
Figures in parentheses indicate the accession number

Fig. 3a–c. In-situ hybridization of IR24 and its two trisomic lines. **a**, IR24; **b**, trisomic line for chromosome 9; **c**, trisomic line for chromosome 10. Bar indicates 3 μm

signals were located at the ends of the signal-tagged chromosomes. The trisomic for chromosome 9 displayed five signals: three large and two medium (Fig. 3b), whereas the trisomic for chromosome 10 showed two large- and three medium-sized signals (Fig. 3c). The signal on chromosome 9 was always larger and the intensity stronger than that on chromosome 10. For the third locus, the chromosome involved could not be definitely determined as there was no trisomic line for this chromosome.

Discussion

In situ hybridization is an effective method for localizing specific RNA or DNA sequences on the chromosomes. It has been applied using radioisotope-labelled ribosomal DNA or RNA (Appels et al. 1980; Gerlach and Peacock 1980; Hutchinson and Miller 1982). Successful mapping or rDNA on rice chromosomes was first achieved using ^{125}I-labelled rDNA probes (Fukui et al. 1987). The resolution of the radioactive probes, however, is limited, especially in small plant chromosomes such as those of rice. It also takes a long time to detect the signals by micro-autoradiography (Fukui 1984).

A non-radioactive labelling method with biotin was developed and widely applied to the ISH method (Rayburn and Gill 1985; Heslop-Harrison 1990; Mukai et al. 1990). The rDNA loci of rice were localized using biotinylated probes (Fukui 1990; Islam-Faridi et al. 1990; Iijima et al. 1991).

Mainly due to the difficulty in preparing chromosomes suitable for ISH and the identification of rice chromosomes after ISH, it has been difficult to localize the genes by ISH in rice, although some positive results have been reported (Wu et al. 1986, 1991; Fukui et al. 1987; Fukui 1990; Suzuki et al. 1991; Gustafson and Dillé 1992).

We have made three improvements in the fluorescence ISH (FISH) procedure to obtain reproducibly clear signals on rice chromosomes. Firstly, a combination of post-treatments was introduced to avoid the thin fluorescent layer often observed to cover all the field after FISH treatment, especially under B excitation light. The four post-treatments applied after sample preparation were as follows: (1) digestion of the polysaccharide layer, originating mainly from the debris of the cell walls, by the use of an enzymatic cocktail; (2) removal of chromosomal proteins by proteinase treatment; (3) elimination of the scattered cytoplasmic debris around the chromosomes by washing with 45% acetic acid, which resulted in a considerable reduction of the noise caused by non-specific signals associated with the fragments of cellular debris; and (4) removal of ribonucleoproteins, that are loosely associated with the surface of the metaphase chromosomes (Sato et al. 1988), by RNase treatment.

Secondly, a rearranged thermal cycler was employed throughout the denaturation process of the chromosomal and the probe DNA to facilitate hybridization of the chromosomal DNA with the probes. The most critical step of FISH is the denaturation process of DNA. Usually this step lasts only a few minutes depending on the material. The maintenance of a constant temperature is rather difficult after the glass slides are dipped in a denaturing solution since the heat capacity of glutaraldehyde is limited (data not shown). The thermal cycler affords the most precise heat control since it was developed for the polymerase chain reaction. Thus the fluctuations caused by manual dipping and raising of the glass slide in and out the solutions were practically eliminated.

Thirdly, an imaging method was introduced to analyze the FISH signal. Faint fluorescent signals, observed especially in B excitation light, could be enhanced and integrated into a chromosomal image in G light by the imaging method. The utility of imaging

methods in plant chromosome research was first revealed in 1985 when a chromosome image analyzing system, CHIAS, was developed (Fukui 1985, 1986, 1988; Fukui et al. 1989). Image analysis for chromosome research has been effective in the identification and characterization of rice chromosomes (Fukui and Iijima 1991, 1992; Iijima et al. 1991).

Evolutionary significance of the variability in the number of rDNA loci

This study has revealed variability in the number of rDNA loci among the eight diploid and the one tetraploid species within the genus *Oryza*. Such variability is rare in the *Hordeum* and *Triticum* (Dvorak et al. 1989; Mukai et al. 1991), where rDNA sites have been investigated using the ISH technique.

It is worth noting that wild rice species have a full range of variability as regards the number of rDNA loci. However, *O. rufipogon*, a putative ancestor of cultivated rice, has rDNA variability which is similar to that of cultivated rice. Cultivated rice has either one or two rDNA loci: Varieties in temperate regions have one rDNA locus while those in tropical and subtropical regions have two rDNA loci. It appears that there has been selection pressure to reduce the number of rDNA loci under adverse conditions such as the low temperature prevalent in temperate areas.

Javanica is sometimes referred to as a tropical *japonica* since there is evidence to prove its similarity with *japonica* based on RFLP analysis (Wang and Tanksley 1989; Kawase et al. 1991) as well as morphological similarities (Oka 1958; Sato 1987). Two *javanica* varieties showed two pairs of rDNA loci indicating the similarity to *indica*. These results may be explained by the environmental similarity of the areas where both javanica and indica varieties are grown.

The NOR chromosome in species with one NOR had already been determined as no. 9, both morphologically (Kurata and Omura 1978; Fukui and Iijima 1991, 1992) and by ISH (Fukui et al. 1987; Fukui 1990; Iijima et al. 1991). Another NOR chromosome was identified as no. 10 (Islam-Faridi et al. 1990). Using the trisomic lines for chromosome 9 or 10, the signal intensity for each locus was found to be different. The locus on chromosome 9 is stronger than that on chromosome 10. The third rDNA locus was the weakest among the three rDNA loci. Since there are no trisomic series in species having this locus, it is difficult to identify the chromosome bearing this rDNA locus. It is, however, suspected to be chromosome 11, due to the conspicuous secondary constriction in the interstitial region of its long arm (Fukui and Iijima 1991). The occurrence of a fluorescent signal at this locus in the interstitial part of the long arm of a rather small chromosome in the two wild rice species lends support to this hypothesis.

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References

Appels R, Gerlach WL, Dennis ES, Swift H, Peacock WJ (1980) Molecular and chromosomal organization of DNA sequence coding for the ribosomal RNAs in cereals. Chromosoma 78:293–311

Dvorak J, Zhang H-B, Kota RS, Lassner M (1989) Organization and evolution of the 5*s* ribosomal RNA gene family in wheat and related species. Genome 32:1003–1016

Fukui K (1984) In-situ hybridization. In: Uchimiya H, Tanaka K, Sugiura A (eds) Manual for plant genetic engineering. Kodansha Scientific, Tokyo, pp 165–168

Fukui K (1985) Identification of plant chromosomes by image analysis method. The Cell (Tokyo) 17:145–149

Fukui K (1986) Standardization of karyotyping plant chromosomes by a newly developed chromosome image analyzing system (CHIAS). Theor Appl Genet 72:27–32

Fukui K (1988) Analysis and utility of chromosome information by using the chromosome image analyzing system, CHIAS. Bull Natl Inst Agrobiol Resour 4:153–175

Fukui K (1990) Localization of rRNA genes on rice chromosomes. Rice Biotech Quart 1:18–19

Fukui K, Iijima K (1991) Somatic chromosome map of rice by imaging methods. Theor Appl Genet 81:589–596

Fukui K, Iijima K (1992) Manual on rice chromosomes. (2nd ed) Misc Pub Natl Inst Agrobiol Resour 4:1–25

Fukui K, Ito K (1989) Computer simulation for microscopic images of barley chromosomes. Bull Natl Inst Agrobiol Resour 5:21–35

Fukui K, Kakeda K (1990) Quantitative karyotyping of barley chromosomes by image analysis methods. Genome 33:450–458

Fukui K, Kamisugi Y (1991) Simulation of microscopic images by a desktop imaging system. In: Doyama M, Suzuki T, Yamamoto R (eds) Computer aided innovation of new materials. Elsevier Science Publishers BV, Amsterdam, pp 101–104

Fukui K, Kakeda K, Hashimoto J, Matsuoka S (1987) In-situ hybridization of ^{125}I-labelled rRNA to rice chromosomes. Rice Genet Newslett 4:114–116

Fukui K, Kadowaki K, Harada K (1989) Implication of the image analysis methods in biotechnology. Jpn Agr Res Quart 22:253–259

Gerlach WL, Peacock WJ (1980) Chromosomal location of highly-repeated DNA sequences in wheat. Heredity 44: 269–276

Gustafson JP, Dillé JE (1992) Chromosome location of *Oryza sativa* recombination linkage groups. Proc Natl Acad Sci USA 89:8646–8650

Heslop-Harrison JS (1990) Gene expression and parental dominance in hybrid plants. Development (suppl): 21–38

Hutchinson J, Miller TE (1982) The nucleolar organizers of tetraploid and hexaploid wheats revealed by in-situ hybridization. Theor Appl Genet 61:285–288

Iijima K, Kakeda K, Fukui K (1991) Identification and characterization of somatic rice chromosomes by imaging methods. Theor Appl Genet 81:597–605

Islam-Faridi MN, Ishii T, Kumar V, Sitch LA, Brar DS (1990) Chromosomal location of ribosomal RNA genes in rice by in-situ hybridization. Rice Genet Newslett 7: 143–144

Kawase M, Kishimoto N, Tanaka T, Yoshimura A, Yoshimura S, Saito K, Saito A, Yano M, Takeda N, Nagamine T, Nakagahra M (1991) Intraspecific variation and genetic differentiation based on restriction fragment length polymorphism in Asian cultivated rice, *Oryza sativa* L. In: IRRI (ed) Rice genetics II, pp 467–473

Khush GS, Kinoshita T (1991) Rice karyotype, marker genes, and linkage groups. In: Khush GS, Toennieson GH (eds), Rice biotechnology. CAB International, Oxford, England, pp 83–108

Kurata N, Omura T (1978) Karyotype analysis in rice. A new method for identifying all chromosome pairs. Jpn J Genet 53:251–255

Leitch IJ, Heslop-Harrison JS (1992) Physical mapping of the 18*s*-58*s*-26*s* rDNA genes in barley by in-situ hybridization. Genome 35:1013–1018

Mukai Y, Endo TR, Gill BS (1990) Physical mapping of the 5*s* rRNA multigene family in common wheat. J Hered 81: 290–295

Mukai Y, Endo TR, Gill BS (1991) Physical mapping of the *18s.26s* rRNA multigene family in common wheat: Identification of a new locus. Chromosoma 100:71–78

Oka HI (1958) Intervariatal variation and classification of cultivated rice. Indian J Genet Plant Breed 18:79–89

Oka HI (1983) Life-history characteristics and colonizing success in plants. Am Zool 23:99–109

Rayburn AL, Gill BS (1985) Use of biotin-labeled probe to map specific DNA sequences on wheat chromosomes. J Hered 76:78–81

Sano Y, Sano R (1990) Variation of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species. Genome 33:209–218

Sato S, Willson C, Dickinson HG (1988) Origin of nucleolus-like bodies found in the nucleoplasm and cytoplasm of *Vicia faba* meristematic cells. Biol Cell 64:321–329

Sato YI (1987) Character association patterns among japonica rice varieties of Asian origin. In: Hsieh SC (ed) Crop exploration and utilization of genetic resources. Taichung Agric Imp Sta, Taiwan, pp 21–29

Suzuki H, Futsuhara Y, Takaiwa F, Kurata N (1991) localization of glutelin gene in rice chromosome by in-situ hybridization. Jpn J Genet 66:305–312

Wang ZY, Tanksley SD (1989) Restriction fragment length polymorphism in *Oryza sativa* L. Genome 32:1113–1118

Wu HK, Chung MC, Xie Y, Wu R (1986) Chromosomal localization of rice *rbsS* genes. Rice Genet Newslett 3:117–119

Wu HK, Chung MC, Chen MH (1985) Karyotype analysis of cultivar IR36. Rice Genet Newslett 2:54–57

Wu HK, Chung MC, Wu T, Ning CN, Wu R (1991) Localization of specific repetitive DNA sequences in individual rice chromosomes. Chromosoma 100:330–338

Yanagisawa T, Tano S, Fukui K, Harada K (1991) Marker chromosomes commonly observed in the genus *Glycine*. Theor Appl Genet 81:606–612

Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes

I. J. LEITCH, A. R. LEITCH, AND J. S. HESLOP-HARRISON

Karyobiology Group, Cell Biology Department, John Innes Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH, United Kingdom

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Two haptens, biotin and digoxigenin, were used to label two highly repetitive plant DNA sequences: pTa71 (a clone containing a ribosomal DNA sequence from wheat, *Triticum aestivum*) and pSc119.2 (a clone containing a 120-bp tandem repeat sequence from rye, *Secale cereale*). These probes were simultaneously localized by *in situ* hybridization on chromosome spreads of rye, *Secale cereale* cv. Petkus Spring. The ability to localize two sequences simultaneously will be of major importance for physically ordering DNA sequences along plant chromosomes.

Key words: physical mapping, DNA–DNA *in situ* hybridization, *Secale*, fluorescent mapping, multiple labelling.

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Deux petites sections détachables (« haptens »), une de chacune des substances biotine et digoxigénine, ont été utilisées pour marquer deux séquences hautement répétitives d'ADN végétal, soit celle de pTa71 (un clone contenant une séquence d'ADN ribosomique de blé, *Triticum aestivum*) et celle de pSc119.2 (un clone comportant une séquence répétitive en tandem de 120 paires de bases de seigle, *Secale cereale*). Ces sondes ont été localisées simultanément par hybridation *in situ* sur des étalements chromosomiques de seigle, *Secale cereale* cv. Petkus Spring. La possibilité de localiser deux séquences simultanément devrait s'avérer d'importance majeure pour déterminer l'agencement physique des séquences d'ADN dans les chromosomes de végétaux.

Mots clés : dressage de cartes physiques, hybridation *in situ* d'ADN–ADN, *Secale*, cartographie par fluorescence, marquages multiples.

[Traduit par la rédaction]

Introduction

Nonradioactive *in situ* hybridization of labelled nucleic acid probes to chromosomal DNA permits the identification of single-copy nucleic acid sequences (Ambros *et al.* 1986), chromosome segments (Le *et al.* 1989; Heslop-Harrison *et al.* 1990), whole chromosomes (Lichter *et al.* 1990), and whole genomes (Le *et al.* 1989; Schwarzacher *et al.* 1989; Leitch *et al.* 1990) at all stages of the cell cycle. Since a nucleic acid probe can be labelled with a variety of different nonradioactive haptens, each of which can be visualized with an independent detection system, it is now possible to detect two (Hopman *et al.* 1986; Cremer *et al.* 1988*a*) or three (Cremer *et al.* 1988*b*; Nederlof *et al.* 1989) DNA sequences simultaneously on animal chromosomes.

The ability to locate several plant DNA sequences simultaneously is important for the physical mapping of plant genomes. However, the technique for gene mapping in plants has lagged behind that for animal chromosome mapping probably as a result of problems associated with the plant cell wall and cytoplasmic material. By first digesting the cell walls with enzymes, using methods developed by Schwarzacher *et al.* (1980), some of these problems are circumvented (Ambros *et al.* 1986). In this paper we demonstrate the simultaneous detection and localization of two highly repeated DNA sequences: pTa71 (a clone containing a ribosomal DNA sequence isolated from wheat) and pSc119.2 (a clone containing a 120-bp tandem repeat sequence isolated from rye). The method involved labelling one probe with biotin and the other with digoxigenin and then using these probes together in an *in situ* hybridization experiment on chromosome spreads of *Secale cereale* cv. Petkus Spring.

Materials and methods

Plant material and chromosome preparation

Root tips were obtained from seedlings of *Secale cereale* L. cv. Petkus Spring. Seeds were germinated on moist filter paper for 48 h at 25°C, 24 h at 4°C, then 26 h at 25°C to synchronize cell divisions. The seedlings were then transferred to 0.05% (w/v) colchicine for 24 h at 4°C to accumulate metaphases, and fixed in ethanol – acetic acid (3:1, v/v). Chromosome spreads were made from enzyme-softened root tips squashed in 45% acetic acid (see Schwarzacher *et al.* 1980).

DNA probes

pTa71 contains a 9-kb *Eco*RI fragment of the ribosomal DNA (rDNA) isolated from wheat, *Triticum aestivum* (Gerlach and Bedbrook 1979), recloned into pUC19 and provided by R.A. Flavell and M. O'Dell; it contains the 5.8S, 18S, 25S, and nontranscribed spacer sequences.

pSc119.2 (kindly supplied by R. Appels; McIntyre *et al.* 1990) was subcloned from pSc119 and includes a 120-bp tandem repeat unit of DNA isolated from rye, *Secale cereale* (Bebrook *et al.* 1980).

One cloned DNA sequence was labelled with biotin-11-dUTP (Sigma) and the other with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation. Immediately prior to *in situ* hybridization both probes were mixed (each to a final concentration of 5 $\mu g\ mL^{-1}$) in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulphate), and 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate).

Pretreatment

Slide preparations were incubated in 100 $\mu g\ mL^{-1}$ DNase-free RNase in 2× SSC for 1 h at 37°C, washed twice in 2× SSC for

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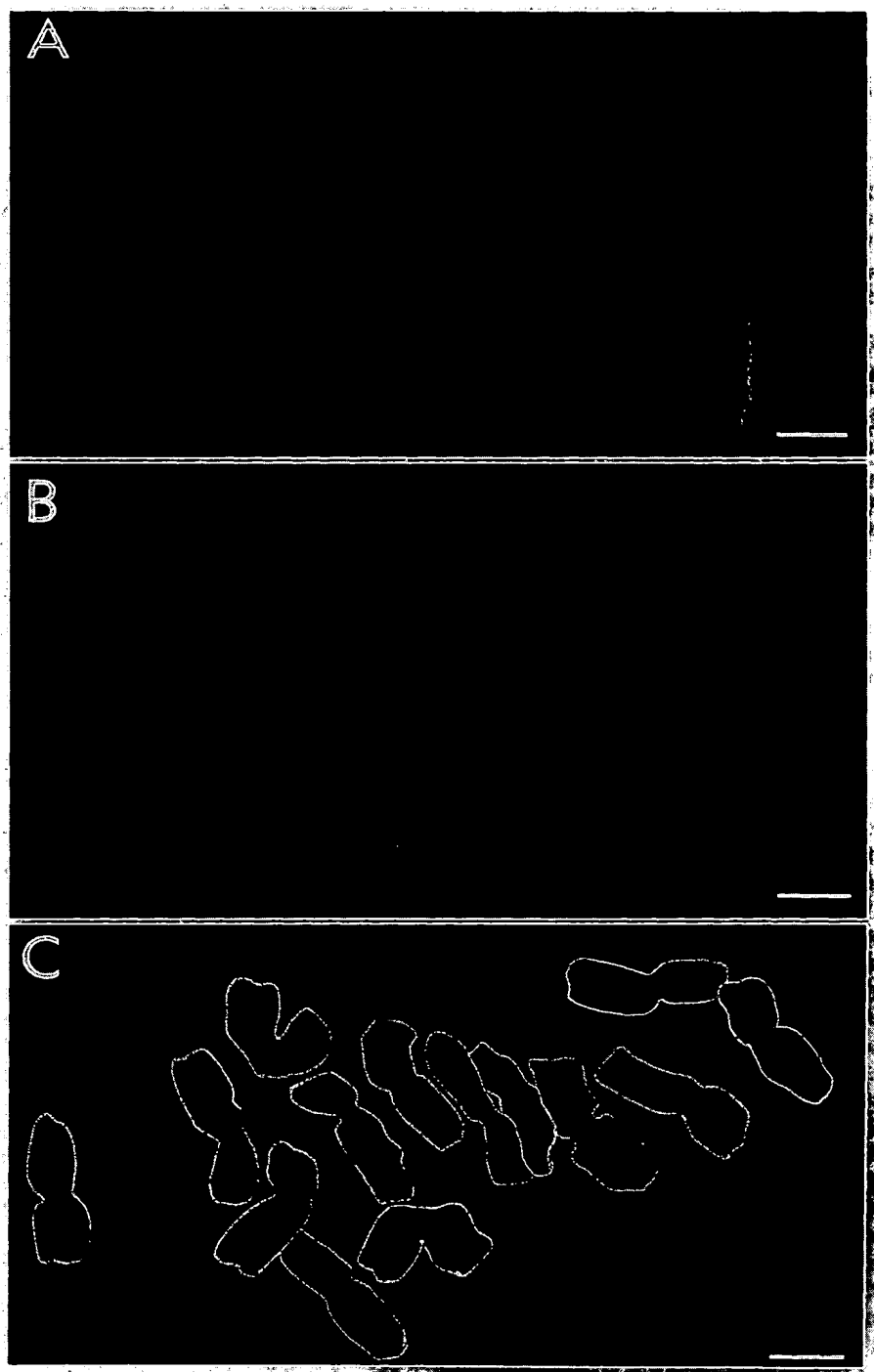


10 min at room temperature, dehydrated in a graded ethanol series, and air dried.

Chromosome and probe denaturation

The probe mixture was denatured at 70°C for 10 min, loaded onto the slide preparation, and covered with a plastic cover slip. The slides were then placed in a humid chamber and the chromosomes and probe were denatured together at 90°C for 10 min.

DNA–DNA hybridization and posthybridization washes

Hybridization was carried out overnight at 37°C. After hybridization, slides were washed in 2× SSC twice, 5 min each, at 40°C and then given a stringent wash in 50% (v/v) formamide in 2× SSC for 10 min at 40°C. The wash allows DNA sequences with more than 85% homology to the probe to remain hybridized, assuming that parameters affecting probe hybridization in solution (Meinkoth and Wahl 1984) are identical for chromosomal DNA *in situ*. The slides were then washed in 2× SSC twice, 5 min each at 40°C and then twice, 5 min each, at room temperature.

Detection of hybridization to cell spreads

The detection of biotin with Texas red labelled avidin (Vector Laboratories) and digoxigenin with sheep anti-digoxigenin-fluorescein (Boehringer Mannheim) was carried out simultaneously. Slides were transferred into detection buffer (4× SSC, 0.2% (v/v) Tween-20) for 5 min, treated with 5% (w/v) BSA (bovine serum albumin) in detection buffer for 5 min, and then incubated in 5 μg mL^{-1} Texas red labelled avidin and 20 μg mL^{-1} anti-digoxigenin-fluorescein in detection buffer containing 5% (w/v) BSA for 1 h at 37°C. After incubation the slides were washed in detection buffer three times, 8 min each, at 37°C.

The signals from biotin and digoxigenin were amplified. After a 5-min block with 5% (v/v) normal goat serum (Vector Laboratories) in detection buffer the slides were incubated with 25 μg mL^{-1} biotinylated anti-avidin D (Vector Laboratories) and 10 μg mL^{-1} FITC-conjugated rabbit anti-sheep (Dakopatts) in detection buffer containing 5% (w/v) normal goat serum for 1 h at 37°C. After washing in detection buffer three times, 8 min each, at 37°C the slides were reincubated with Texas red labelled avidin as described above.

The slides were counterstained with 2 μg mL^{-1} DAPI (4′, 6-diamidino-2-phenylindole) in McIlvaine's citrate buffer (0.01 M citric acid, 0.08 M sodium hydrogen phosphate, pH 7) and then were mounted in antifade solution (AFI, Citifluor) to reduce fading of fluorescence. Slides were examined with a Zeiss epifluorescence microscope with filter sets 02, 09, and 12. Photographs were taken on Fujicolor Super HG 400 colour print film.

Results

Root-tip metaphase spreads of *S. cereale* were stained with the fluorochrome DAPI, which fluoresces blue (emission maximum, 455 nm) on excitation with ultraviolet light (excitation maximum, 365 nm) (Figs. 1A and 2A). DAPI, which quantitatively binds to AT-rich sequences (Schweizer 1981), showed nonuniform fluorescence along the chromosomes. Following *in situ* hybridization with digoxigenin labelled pSc119.2, detected by FITC fluorescence (excitation maximum, 495 nm (blue); emission maximum, 515 nm (green)), and biotinylated pTa71 detected by Texas red fluorescence (excitation maximum, 595 nm (green); emission maximum, 615 nm (red)), both sequences could be mapped to *S. cereale* chromosomes simultaneously (Figs. 1B and 1C). Most locations of pSc119.2 hybridization (Fig. 1B) occurred at sites of heterochromatin as revealed by DAPI fluorescence. The location of the 120-bp tandem repeat sequence is known to be correlated with some of the heterochromatic C-bands of rye (Jones and Flavell 1982). The two sites of pTa71 hybridization (Fig. 1C) mapped onto the short arm of chromosome 1R, corresponding to the location of the nucleolar organizing region in rye. As a result of the high copy number of rRNA genes in cereals (estimated to be 5 000 – 18 000 per genome (Flavell and Smith 1974; Liang *et al.* 1977)) Texas red fluorescence was also visible following excitation with blue light for FITC (Fig. 1B). It arises because there is an overlap in the excitation spectra of the two fluorochromes such that detectable Texas red fluorescence occurs when chromosomes are strongly labelled.

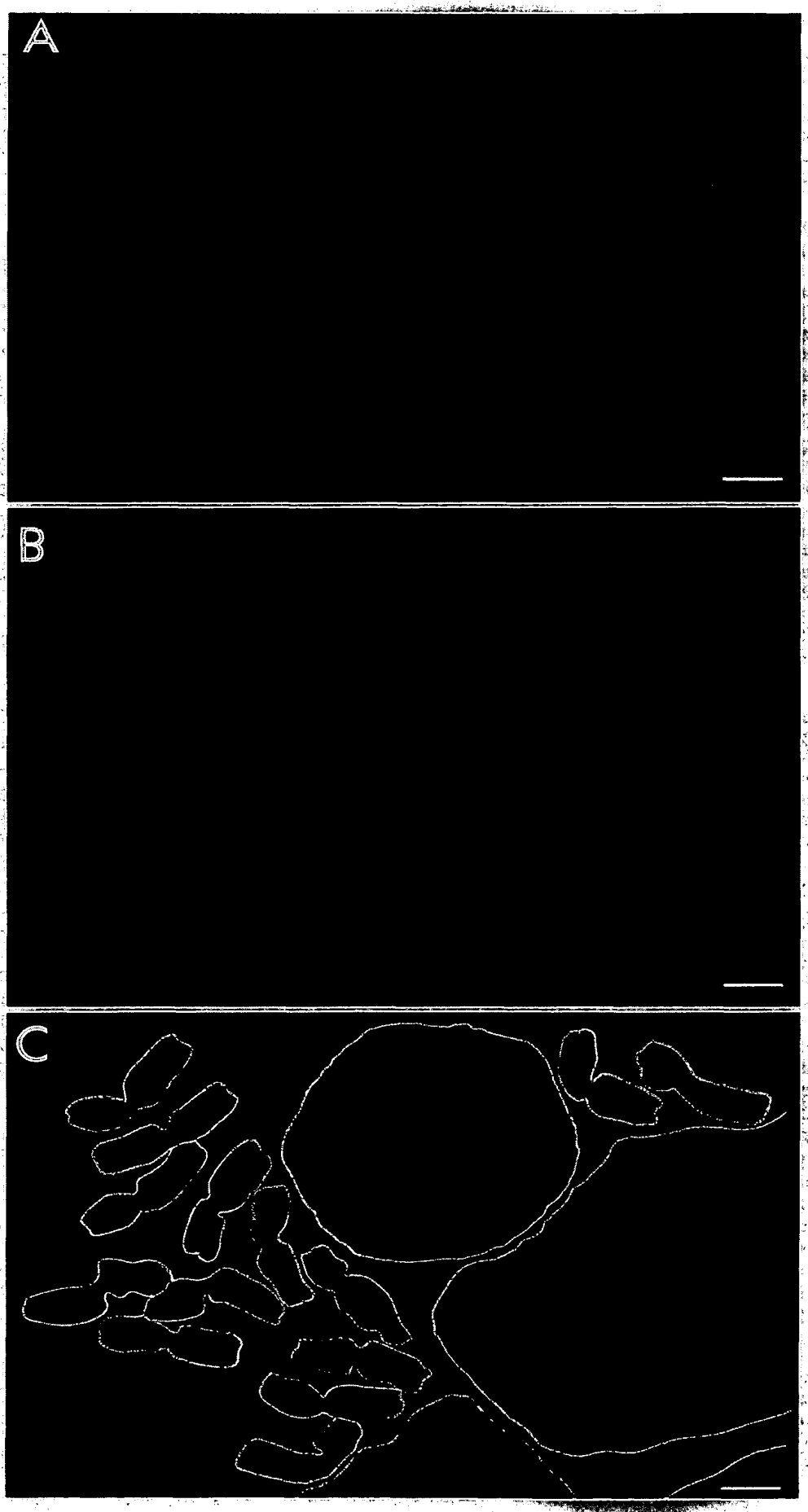
By exchanging the labelling of the probes (i.e., pTa71 labelled with digoxigenin and pSc119.2 labelled with biotin) the sites of Texas red and FITC fluorescence were reversed (Fig. 2). Texas red fluorescence in the region of the rDNA sequences (Fig. 2C), which is substantially less than the signal, is probably due to a limited cross-reactivity of the different antibodies used in the detection steps. Comparison of the two figures shows the sensitivities of detection achieved by the two labelling methods is similar.

Discussion

The ability to localize more than one probe in a single experiment is of major importance for plant molecular cytology. Unlike Southern hybridization experiments, techniques to reprobe chromosome preparations are not available and hence direct comparisons of different probe hybridization sites following *in situ* hybridization cannot be made. Comparing hybridization sites of different probes on different metaphase chromosomes is unsatisfactory because measurements between the probe site and morphological features of chromosomes (including telomeres, centromeres, and nucleolar organizing regions) will differ depending on chromosome condensation and other factors (Lichter *et al.* 1990). *In situ* hybridization experiments using two or more probes simultaneously enables direct ordering of the probe hybridization sites on one metaphase chromosome (Lawrence *et al.* 1990). In addition simultaneous detection of hybridization sites of multiple probes will be useful to determine the direction of chromosome walking in long-range gene cloning experiments.

Visualizing multiple targets simultaneously in a single experiment is of particular importance for the physical mapping of genes along plant chromosomes where chromosome banding patterns (such as C- and N-bands) are limited. By using one probe for the identification of a specific chromosome, additional sequences can be mapped along the chromosome arm without the need to resort to banding patterns for chromosome identification. In theory, the number of sequences that can be ordered along a single chromosome

FIG. 1. Root-tip metaphase cell spread from *S. cereale* after the simultaneous *in situ* hybridization and detection of biotinylated-pTa71 and digoxigenin labelled pSc119.2. (A) DAPI staining for DNA. (B) The hybridization sites of pSc119.2 are detected by yellow FITC fluorescence corresponding to the location of the 120-bp tandem repeat sequence of DNA. The pTa71 signal is also visible (orange bands). (C) The hybridization sites of pTa71 are detected by Texas red fluorescence (red bands) corresponding to the location of the rRNA genes in rye. The chromosome outlines are drawn in white based on the DAPI staining seen in Fig. 1A. Scale bars represent 5 μm.


A
B
C

type can be expressed by $S = L^N$, where S is the number of sequences that can be ordered; L is the number of different labelling and detection systems; and N is the number of slides. By using this equation one could speculate that eight sequences could be mapped in order along a chromosome type by examining only three slides, on each of which the eight probes had been labelled in different combinations with one of two systems. The development of additional labelling-detection combinations will further simplify the determination of gene order and increase the efficiency of physical mapping of DNA sequences.

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AMBROS, P.F., MATZKE, M.A., and MATZKE, A.J.M. 1986. Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. Chromosoma, **94**: 11–18.

BEDBROOK, J.R., JONES, J., O'DELL, M., THOMPSON, R.D., and FLAVELL, R.B. 1980. Molecular characterization of telomeric heterochromatin in *Secale* species. Cell, **19**: 545–560.

CREMER, T., LICHTER, P., BORDEN, J., WARD, D.C., and MANUELIDIS, L. 1988*a*. Detection of chromosomal aberrations in metaphase and interphase tumor cells by *in situ* hybridization using chromosome-specific library probes. Hum. Genet. **80**: 235–246.

CREMER, T., TESIN, D., HOPMAN, A.H.N., and MANUELIDIS, L. 1988*b*. Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumour cells by *in situ* hybridization with chemically modified DNA probes. Exp. Cell Res. **176**: 199–220.

FLAVELL, R.B., and SMITH, D.B. 1974. Variation in nucleolar organiser rRNA gene multiplicity in wheat and rye. Chromosoma, **47**: 327–334.

GERLACH, W.L., and BEDBROOK, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

HESLOP-HARRISON, J.S., LEITCH, A.R., SCHWARZACHER, T., and ANAMTHAWAT-JÓNSSON, K. 1990. Detection and characterization of 1B/1R translocations in hexaploid wheat. Heredity, **65**: 385–392.

HOPMAN, A.H.N., WIEGANT, J., RAAP, A.K., LANDEGENT, J.E., VAN DER PLOEG, M., and VAN DUIJN, P. 1986. Bi-color detection of two target DNAs by nonradioactive *in situ* hybridization. Histochemistry, **85**: 1–4.

JONES, J.D.G., and FLAVELL, R.B. 1982. The mapping of highly-repeated DNA families and their relationship to C-bands in chromosomes of *Secale cereale*. Chromosoma, **86**: 595–612.

LAWRENCE, J.B., SINGER, R.H., and MCNEIL, J.A. 1990. Interphase and metaphase resolution of different distances within the human dystrophin gene. Science (Washington, D.C.), **249**: 928–932.

LE, H.T., ARMSTRONG, K.C., and MIKI, B. 1989. Detection of rye DNA in wheat-rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Mol. Biol. Rep. **7**: 150–158.

LEITCH, A.R., MOSGÖLLER, W., SCHWARZACHER, T., BENNETT, M.D., and HESLOP-HARRISON, J.S. 1990. Genomic *in situ* hybridization to sectioned nuclei shows chromosome domains in grass hybrids. J. Cell Sci. **95**: 335–341.

LIANG, G.H., WANG, A.S., and PHILLIPS, R.L. 1977. Control of ribosomal RNA multiplicity in wheat. Can. J. Genet. Cytol. **19**: 425–435.

LICHTER, P., TANG, C.-J.C., CALL, K., HERMANSON, G., EVANS, G.A., HOUSMAN, D., and WARD, D.C. 1990. High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science (Washington, D.C.), **247**: 64–69.

MCINTYRE, C.L., PEREIRA, S., MORAN, L.B., and APPELS, R. 1990. New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. Genome, **33**: 635–640.

MEINKOTH, J., and WAHL, G. 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**: 267–284.

NEDERLOF, P.M., ROBINSON, D., ABUKNESHA, R., WIEGANT, J., HOPMAN, A.H.N., TANKE, H.L., and RAAP, A.K. 1989. Three-color fluorescence *in situ* hybridization for the simultaneous detection of multiple nucleic acid sequences. Cytometry, **10**: 20–27.

SCHWARZACHER, T., AMBROS, P., and SCHWEIZER, D. 1980. Application of Giemsa banding to orchid karyotype analysis. Plant Syst. Evol. **134**: 293–297.

SCHWARZACHER, T., LEITCH, A.R., BENNETT, M.D., and HESLOP-HARRISON, J.S. 1989. *In situ* localization of parental genomes in a wide hybrid. Ann. Bot. (London), **64**: 315–324.

SCHWEIZER, D. 1981. Counterstain-enhanced chromosome banding. Hum. Genet. **57**: 1–14.

FIG. 2. Root-tip metaphase cell spread from *S. cereale* after the simultaneous *in situ* hybridization and detection of biotinylated-pSc119.2 and digoxigenin labelled pTa71. (A) DAPI staining for DNA. (B) The sites of hybridization of pTa71 are detected by FITC fluorescence (yellow bands) corresponding to the location of rRNA genes in rye. The sites of pSc119.2 hybridization are also visible, weakly fluorescing orange. (C) The hybridization sites of pSc119.2 are detected by Texas red fluorescence corresponding to the location of the 120-bp tandem repeat sequence of DNA in rye. Texas red fluorescence in the region of the rDNA sequences is probably due to a limited cross-reactivity between the different antibodies used in the detection. The chromosome outlines are drawn in white based on the DAPI staining seen in Fig. 2A. Scale bars represent 5 μm.

REVIEW / SYNTHÈSE

Nonisotopic in situ hybridization and plant genome mapping: the first 10 years

JIMING JIANG[1] AND BIKRAM S. GILL[2]

Wheat Genetics Resource Center and Department of Plant Pathology, Kansas State University, Manhattan, KS 66502-5506, U.S.A.

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JIANG, J., and GILL, B.S. 1994. Nonisotopic in situ hybridization and plant genome mapping: the first 10 years. Genome, **37**: 717–725.

Nonisotopic in situ hybridization (ISH) was introduced in plants in 1985. Since then the technique has been widely used in various areas of plant genome mapping. ISH has become a routine method for physical mapping of repetitive DNA sequences and multicopy gene families. ISH patterns on somatic metaphase chromosomes using tandemly repeated sequences provide excellent physical markers for chromosome identification. Detection of low or single copy sequences were also reported. Genomic in situ hybridization (GISH) was successfully used to analyze the chromosome structure and evolution of allopolyploid species. GISH also provides a powerful technique for monitoring chromatin introgression during interspecific hybridization. A sequential chromosome banding and ISH technique was developed. The sequential technique is very useful for more precise and efficient mapping as well as cytogenetic determination of genomic affinities of individual chromosomes in allopolyploid species. A critical review is made on the present resolution of the ISH technique and the future outlook of ISH research is discussed.

Key words: in situ hybridization, physical mapping, genome mapping, molecular cytogenetics.

JIANG, J., et GILL, B.S. 1994. Nonisotopic in situ hybridization and plant genome mapping: the first 10 years. Genome, **37** : 717–725.

L'hybridation in situ (ISH) non-isotopique a été utilisée chez les plantes pour la première fois en 1985. Depuis lors, la technique a été employée largement dans plusieurs domaines de la cartographie des génomes végétaux. L'hybridation in situ est devenue une technique de routine dans la cartographie physique des séquences d'ADN répétées et des familles de gènes présents en copies multiples. Les motifs produits sur les chromosomes somatiques en métaphase suite à l'hybridation in situ de séquences répétées en tandem constituent d'excellents marqueurs physiques aux fins d'identification des chromosomes. La détection de séquences uniques ou en faibles copies a également été rapportée. L'hybridation in situ génomique (GISH) a été employée avec succès dans l'analyse de la structure des chromosomes et de l'évolution des espèces allopolyploïdes. L'hybridation in situ génomique constitue une technique puissante pour suivre le degré d'introgression obtenu lors de croisements interspécifiques. Une technique séquentielle de striation chromosomique (« chromosome banding ») suivie d'hybridation in situ a été mise au point. Cette technique permet une cartographie plus précise et plus efficiente de même qu'une détermination cytogénétique des affinités génomiques de chromosomes individuels à l'intérieur d'espèces allopolyploïdes. Les auteurs présentent un examen critique de la résolution présentement offerte par l'hybridation in situ et discutent des avenues de recherche futures de cette technique.

Mots clés : hybridation in situ, cartographie physique, cartographie génomique, cytogénétique moléculaire.

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[1]Present address: Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510, U.S.A.
[2]Author to whom all correspondence and reprint requests should be addressed.

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Introduction

The in situ hybridization (ISH) technique, developed more than 20 years ago (Gall and Pardue 1969; John et al. 1969), provides a method to localize genes or DNA sequences directly on chromosomes in cytological preparations. The original ISH technique using isotopic probes is sensitive and very useful for detecting single-copy DNA sequences (see review by McNeil et al. 1991). The main disadvantages of the isotopic ISH technique are the limited resolution owing to the use of an emitted isotopic signal captured by an emulsion layer and the need for scoring and statistical analysis of silver grains on a large number of metaphase cells. Moreover, the detection of hybridization sites by the autoradiographic procedure requires a long exposure time of several weeks or months.

To overcome these limitations, nonisotopic ISH techniques were developed (Langer-Safer et al. 1982). Initially, nonisotopic ISH techniques were used for detection of repetitive DNA sequences or amplified sequences in polytene chromosomes only (Langer-Safer et al. 1982; Manuelidis et al. 1982), but since then the sensitivity of the technique has been greatly improved. Detection of single-copy DNA sequences as small as 0.9 kb was reported in humans (Viegas-Péquignot et al. 1991). Moreover, nonisotopic ISH techniques are amenable to fluorescent and confocal microscopic techniques, and image processing technology (Lichter et al. 1990). The present paper will review the applications of nonisotopic ISH techniques in plant genome mapping during the last 10 years.

The basic technique

The nonisotopic ISH technique, using biotin-labelled DNA probes (Langer-Safer et al. 1982; Manuelidis et al. 1982), was first introduced in plant species by Rayburn and Gill in 1985. In this procedure, the probe DNA is labelled with biotinylated dUTP and the hybridization sites are detected by enzymatic reporter molecules, commonly horseradish peroxidase or alkaline phosphatase conjugated avidin/streptavidin. Recently, a system using digoxygenin-labeled nucleotides detected by antibodies carrying enzymatic tags has also become popular. The enzymatic detection methods have advantages over the fluorescence methods (see below) in that the reaction can be prolonged to amplify signals and the signals do not fade.

Fluorescence in situ hybridization (FISH), using fluorochromes for signal detection (Langer-Safer et al. 1982; Pinkel et al. 1986), has several advantages over ISH using enzymatic detection methods. First, different DNA probes can be labelled with different haptens and simultaneously detected using different fluorochromes (multicolor FISH), thus allowing their physical order on chromosomes to be determined (Lichter et al. 1990; Leitch et al. 1991; Mukai et al. 1993*a*). If a specific probe having multiple sites that is useful for chromosome identification was previously mapped, a new probe can be directly mapped to a specific chromosome by cohybridization with the known probe (Jiang and Gill 1994*b*). Second, fluorescence signals can be captured by special cameras or laser scanning microscopes and analyzed with digital imaging systems, thus allowing more precise mapping.

One of the important modifications of the ISH technique is genomic in situ hybridization (GISH) (Durnam et al. 1985; Pinkel et al. 1986; Le et al. 1989; Schwarzacher et al. 1989). In GISH, total DNA from the genome of one parent of a polyploid species or a hybrid is labelled as a probe, and unlabelled total DNA from the other genome(s) of other parent(s) is added in the hybridization mixture, resulting in the differentiation of chromosomes from different genomes. The preferential hybridization of the labelled genomic probe to the chromosomes of its genomic origin is most likely due to the presence of genome-specific repetitive DNA sequences. If two genomes are very closely related and share most of the repetitive DNA sequences, then the distinction between the two genomes becomes relatively difficult.

Sequential banding and ISH technique

In humans, ISH is integrated with various chromosome banding techniques into a single procedure, and DNA sequences can be directly mapped to specific chromosomal regions (Lawrence et al. 1990; McNeil et al. 1991). In plants, Hutchinson and Seal (1983) localized a repetitive DNA sequence on individual rye (*Secale cereale*) chromosomes by a sequential isotopic ISH and C-banding procedure. However, the resolution of the C-banding was greatly reduced. No further results were reported on the application of this technique.

To exploit the potential of sequential applications of banding and in situ hybridization on plant chromosomes, we tried different combinations of N- or C-banding with ISH. A modified N-banding/ISH sequential procedure gave the best results. Similarly, a modified C-banding/ISH also gave satisfactory results. The hot acid treatment in the regular N- or C-banding procedures was the major factor affecting the resolution of the subsequent ISH. Therefore, the hot acid treatment was deleted (in N-banding) or modified (in C-banding) to allow subsequent ISH. By the sequential modified N- or C-banding – ISH/GISH techniques, multicopy DNA sequences and the breakpoints of intergenomic translocation chromosomes were directly allocated to specific wheat chromosomes (Jiang and Gill 1993*a*, 1994*a*, 1994*b*) (Fig. 1).

Mapping of repetitive DNA sequences and multicopy gene families

Repetitive DNA sequences

ISH is the most accurate way to determine the dispersed or tandem distribution of repetitive DNA sequences on individual chromosomes. A large number of repetitive DNA sequences from plant species were physically mapped by ISH (Rayburn and Gill 1985, 1986; Lapitan et al. 1989; Maluszynska and Heslop-Harrison 1991; Tsujimoto and Gill 1991; Anamthawat-Jónsson and Heslop-Harrison 1993; Jiang et al. 1994*b*).

Physical locations of various repetitive DNA sequences can be used to analyze the molecular nature of heterochromatin. For example, sequences of the 480-bp family (also named the 350 bp family) from rye (*Secale cereale*) are mainly located at the telomeric regions on all seven pairs of chromosomes (Bedbrook et al. 1980; Appels et al. 1981). These locations correspond to most of the major C-banded regions (Mukai et al. 1992). Several other major repetitive DNA sequence families were dispersed in the entire rye genome (Bedbrook et al. 1980; Appels et al. 1986; Guidet et al. 1991). This was the first systematic analysis of the relationship between different heterochromatic bands and repetitive DNA sequences in plants.

Molecular karyotypes can be constructed by ISH using

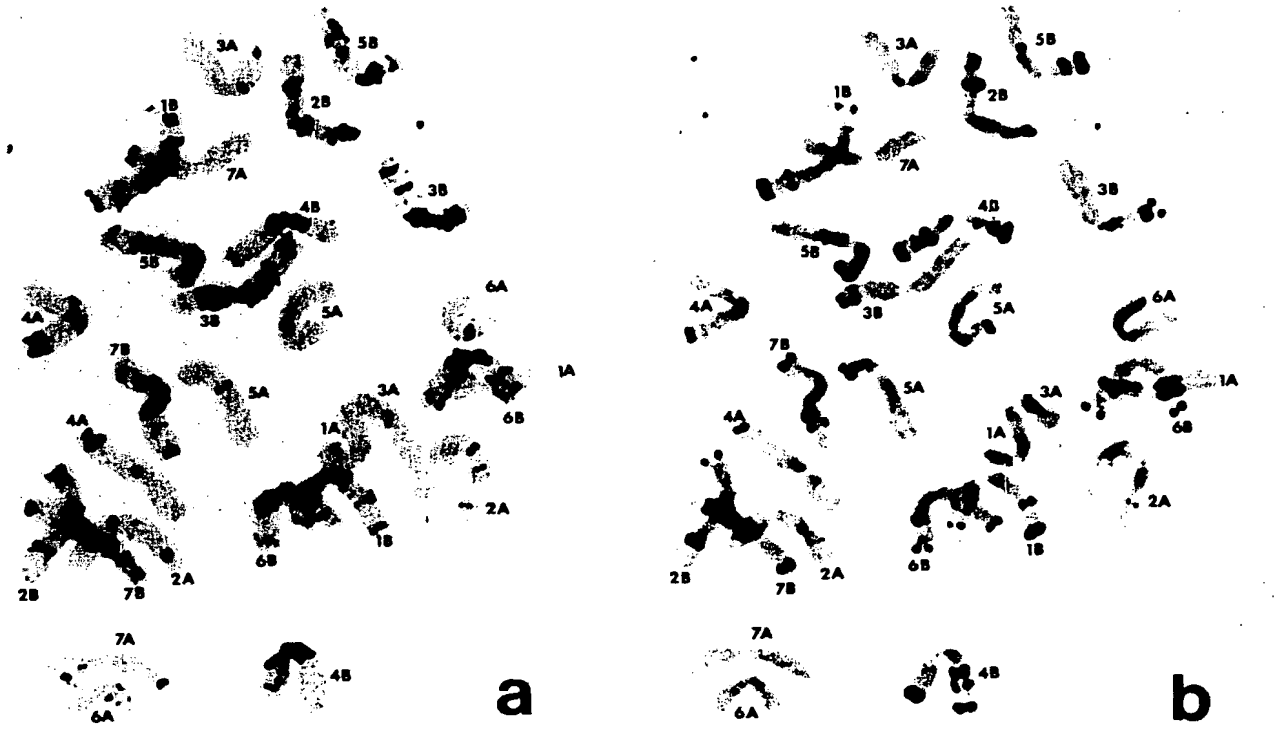

FIG. 1. Sequential N-banding and in situ hybridization using repetitive DNA sequence pSc119 as a probe on metaphase chromosomes of *Triticum turgidum* var. *durum* cv. Langdon. (*a*) N-banding patterns. All 28 chromosomes of cv. Langdon can be identified. (*b*) Subsequent ISH on the same metaphase cell. All B genome chromosomes, together with 4A and 5A, show unique hybridization patterns.

tandemly repeated DNA sequences as probes (Rayburn and Gill 1985, 1986; Lapitan et al. 1989; Mukai et al. 1993*a*). Unique ISH patterns on individual chromosomes provide an alternative method for chromosome identification. For example, the 120-bp repetitive DNA sequence family from rye gave unique ISH patterns on chromosomes from several species of the Triticeae (Rayburn and Gill 1985, 1988; Lapitan et al. 1987; Friebe et al. 1991). All chromosomes from the B and G genomes and several chromosomes from the A and D genomes can be identified by ISH patterns using the 120-bp sequence as a probe (Rayburn and Gill 1985; Jiang and Gill 1994*a*) (Fig. 1). The 18S·26S ribosomal RNA genes were directly mapped on chromosomes of *Aegilops speltoides* by cohybridization with the 120-bp sequence (Jiang and Gill 1994*b*).

Multicopy gene families

ISH was successfully used to map multicopy gene families, such as the 5S and 18S·26S ribosomal genes, on plant chromosomes (Skorupska et al. 1989; Bergey et al. 1989; Mukai et al. 1990, 1991; Griffor et al. 1991; Lapitan et al. 1991; Leitch and Heslop-Harrison 1992, 1993; Ricroch et al. 1992) (Fig. 2*a*). ISH permits not only the physical localization of these multicopy genes but also identification of gene loci lacking polymorphism that otherwise cannot be mapped by conventional RFLP analysis. Several new loci of 5S and 18S·26S rRNA genes in wheat and barley (*Hordeum vulgare*) were detected by ISH analysis (Mukai et al. 1990, 1991; Leitch and Heslop-Harrison 1992, 1993; Jiang and Gill 1994*b*). Multicopy genes were also mapped on specific chromosomes in meiosis (Crane et al. 1993).

Mapping of low- or single-copy sequences: a critical evaluation

A great effort was made in several laboratories to improve the sensitivity of the nonisotopic ISH technique for mapping low- or single-copy DNA sequences on plant chromosomes. However, the resulting reports need to be evaluated carefully. Reports of detection of low- or single-copy sequences included a 17-kb T-DNA in *Crepis capillaris* (Ambros et al. 1986), a 13.5-kb legumin gene in pea (*Pisum sativum*) (Simpson et al. 1988), the β-tubulin gene in alfalfa (Schaff et al. 1990), the B-hordein gene cluster with more than 10 copies in barley (*Hordeum vulgare*) (Lehfer et al. 1993), and the α-amylase-2 gene in barley (Leitch and Heslop-Harrison 1993). In these successful cases, the targeted DNA sequences on chromosomes were all more than 10 kb long.

There are also reports of mapping of DNA sequences as small as 0.7 kb (Gustafson et al. 1990; Gustafson and Dillé 1992). The reported frequency of signal detection was very low (one or more than one ISH signal in 6% of the metaphase cells observed), and usually the signal was detected on one chromatid only. At such low detection frequencies, without a careful monitoring of the target chromosome, the distinction between a true signal and an artifact or a background spot is difficult. For example, Clark et al. (1979) localized the B-hordein gene to 46% of the distance

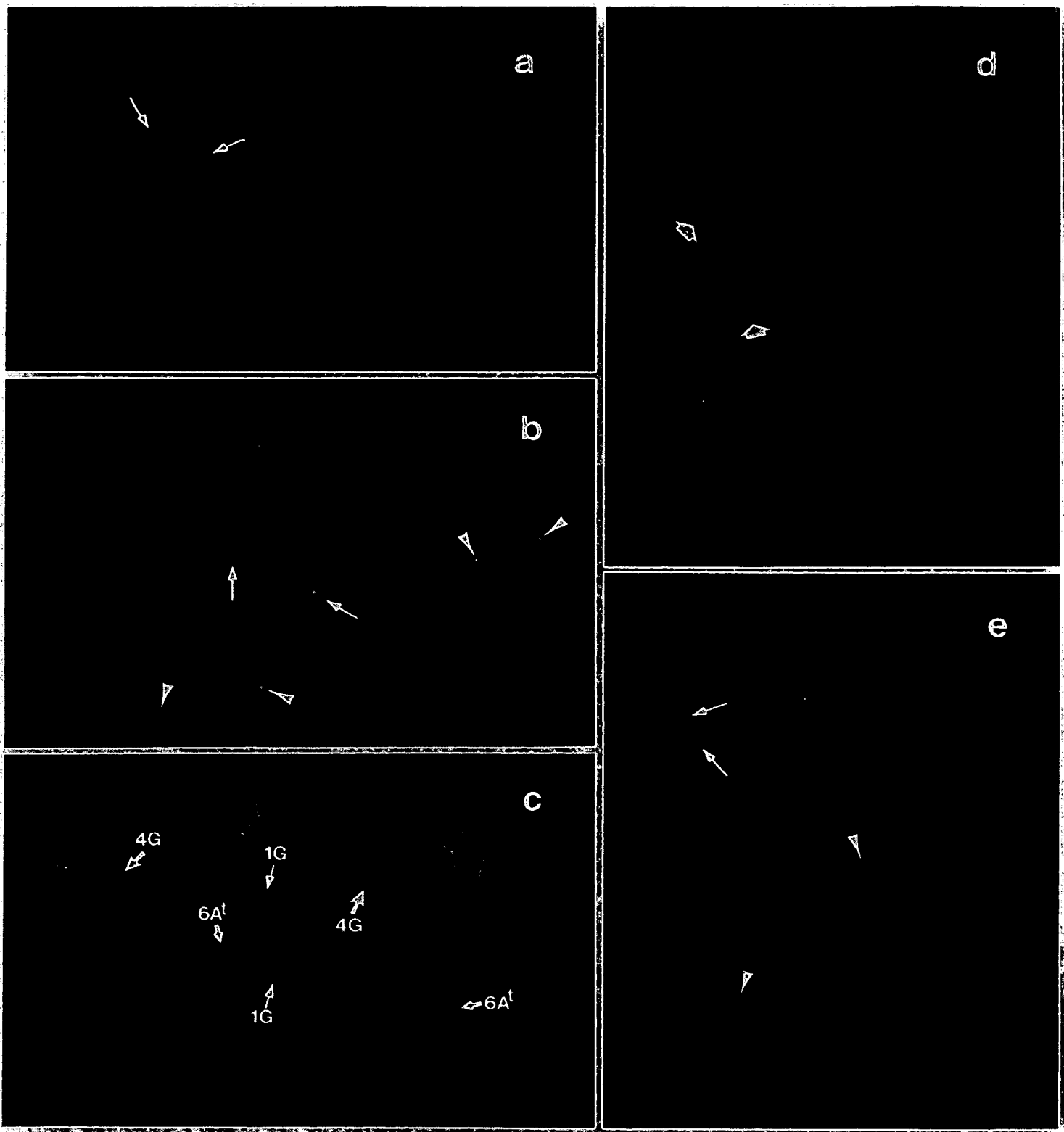

FIG. 2. (*a*) In situ hybridization mapping of the 18S·26S ribosomal RNA genes on metaphase chromosomes of maize. The genes are located at the nucleolar organizer region of chromosome 6 (arrowed). (*b*) In situ hybridization mapping of a genomic clone (*KSU3*) on maize metaphase chromosomes. The probe is located at the terminal region of chromosome 10 (arrows). Hybridization signals are also detected in the interphase nucleus (arrowheads). (*c*) Genomic in situ hybridization analysis of *Triticum timopheevii*. *Triticum monococcum* DNA is used as probe and *Aegilops speltoides* DNA as a block. Three A^t/G intergenomic translocation chromosomes, 1G, 4G, and $6A^t$, are arrowed. For details see Jiang and Gill (1994*a*). (*d*) Genomic in situ hybridization of a wheat – *Elymus trachycaulus* multiple chromosome translocation line z5A. Two chromosomes (arrowed) show a striped hybridization pattern. For details

from the centromere on the short arm of barley chromosome 5 with a detection frequency of 0.025. Lehfer et al. (1993) recently reported that the B-hordein gene is actually located at the distal end of the short arm of chromosome 5. The discrepancy of these two reports is most likely due to the misidentification of the real ISH signals by Clark et al. (1979).

To estimate the sensitivity of the ISH technique, we used a maize (*Zea mays*) genomic clone (*KSU3*) as a probe. *KSU3* contains a fragment about 3.5 kb long and was genetically mapped on the short arm of chromosome 10 (Hong et al. 1993). This fragment is amplified as tandem clusters with different copies in different maize lines (Hong et al. 1993). In a line with about 10 copies of the protein kinase gene, the ISH signals using this gene as a probe can be clearly located in the distal region of the short arm of the smallest maize chromosome (chromosome 10). The signals appeared as single dots on both chromatids of both homologous chromosomes (Fig. 2*b*) and can be detected in more than 90% of the cells. However, in other lines with only one or few copies of the gene, ISH signals become much weaker compared with those on the first line, and the frequency of signal detection becomes very low.

Genome analysis and monitoring of alien chromatin

Genome analysis

Genomes that are genetically very close, such as those from barley and *Hordeum bulbosum*, can be differentiated by GISH (Anamthawat-Jónsson et al. 1993). Thus, GISH can be applied in chromosome pairing analysis to monitor intergenomic (homoeologous) pairing (Le and Armstrong 1991; King et al. 1993; Chen et al. 1994). It is difficult to distinguish intragenomic and intergenomic chromosome pairing by conventional meiotic analysis. GISH is thus a valuable supplemental technique to the traditional genome analysis method.

GISH was successfully used to analyze the chromosome structure in allopolyploid species. GISH analysis using genomic DNA from probable diploid progenitor species as probes provided important information of the genome evolution and divergence of allopolyploid species (Bennett et al. 1993; Kenton et al. 1993; Mukai et al. 1993*a*; Jiang and Gill 1994*a*; Jellen et al. 1994). A number of intergenomic translocations were discovered in tobacco (*Nicotiana tabacum*, SSTT) genomes (Kenton et al. 1993). Similarly, GISH was used to identify intergenomic A–C and A/D–C translocations in allotetraploid and hexaploid oat species (genus *Avena*) (Jellen et al. 1994; Chen and Armstrong 1994). A species-specific cyclic translocation involving chromosomes 1G, 4G, and 6A^t was found in *Triticum timopheevii* (A^{t}A^{t}GG) by GISH analysis (Jiang and Gill 1994*a*) (Fig. 2*c*). A different cyclic translocation involving chromosomes 4A, 5A, and 7B was reported in *Triticum turgidum* and *Triticum aestivum* (Naranjo et al. 1987; Naranjo 1990; Liu et al. 1992). The presence of different species-specific chromosome translocations, together with the cytoplasmic difference of the A^{t}A^{t}GG and AABB species, support the theory that the polyploid A^{t}A^{t}GG and AABB species were derived from two different original amphiploids (Tsunewaki and Ogihara 1983; Jiang and Gill 1994*a*).

If the morphology of chromosomes from different genomes in a polyploid species have no distinct differences, the determination of the genomic affinity of individual chromosomes is very difficult. The pioneering work was done in hexaploid wheat by chromosome pairing analysis using aneuploids (Sears 1954; Okamoto 1962). However, this strategy can hardly be applied to other polyploid species because of the time-consuming work of aneuploid isolation. A rapid method to assign individual chromosomes to different genomes in a polyploid species was developed by a sequential banding and GISH analysis (Jiang and Gill 1994*a*). Chromosomes were identified by either modified N- or C-banding. Well-spread and well-banded metaphase cells were photographed and the preparations then were analyzed with GISH. By comparing the banding and GISH photographs from the same metaphase cell, a group of banded chromosomes can be assigned to the labelled genome.

Monitoring of alien chromatin

Introgression of alien chromosome segments containing useful genes into crop plants through wide hybridization is a valuable method for plant breeding. As a good example, the short arm of rye chromosome 1R, carrying several disease resistance genes, was incorporated into many high-yielding wheat cultivars (Rajaram et al. 1983). Monitoring of alien chromatin during introgression is critical for a successful transfer.

A number of different techniques can be used to detect alien chromatin (see review by Jiang et al. 1994*a*). GISH is the most efficient and accurate technique to allocate the breakpoints and estimate the amount of alien chromatin of the translocation chromosomes. Although ISH using highly dispersed, species-specific repetitive DNA sequences as probes can also be used to detect the breakpoints of the translocation chromosomes (Lapitan et al. 1986; Friebe et al. 1993*a*; Kim et al. 1993*a*, 1993*b*), isolation of such types of DNA sequences is difficult and time consuming. Many wheat–alien chromosome translocation lines were characterized by GISH analysis (see review by Jiang et al. 1994*a*; Friebe et al. 1994*b*) (Figs. 2*d* and 2*e*). Chromosome banding is required to identify the wheat chromosomes involved in the translocations (Friebe et al. 1992, 1993*b*, 1994*a*; Jiang and Gill 1993*b*; Jiang et al. 1993; Jiang et al. 1994*b*; Mukai et al. 1993*b*). Sequential banding and GISH permits direct identification of the translocation chromosomes (Jiang and Gill 1993*a*). The combination of chromosome banding and GISH also allows the estimation of the size of the added alien chromosome segment and the missing wheat chromosome segment (Friebe et al. 1992, 1993*b*, 1994*a*; Jiang et al. 1993).

Other applications

GISH was applied to analyze the distribution of chromatin from different genomes in interspecific hybrids. Analysis of interphase nuclei of a *Hordeum chilense* × *Secale africanum* hybrid showed that chromatin originating from the parental genomes did not intermix but occupied distinct domains (Leitch et al. 1990). Similar separation of parental

see Jiang and Gill (1993*b*). (*e*) Chromosome painting of cv. Amigo wheat. The rye chromosome arm 1RS on translocation chromosomes T1AL·1RS is detected by Rodamine (red color, arrowheads). The *Agropyron elongatum* chromosome segment in cv. Amigo was first reported by The et al. (1992). This *Agropyron elongatum* segment is located on the satellite of chromosome 1B (J. Jiang and B.S. Gill, unpublished results) and is detected by FITC (turquoise color, arrows). Chromosomes were counterstained by DAPI.

genomes throughout the cell cycle was also observed in a *H. vulgare* × *S. africanum* hybrid (Leitch et al. 1991). These results indicated that the hybrid nuclei are spatially organized at the genome level.

Separation of a chromosome from the rest of the genome in interphase nuclei was also observed in wheat–alien chromosome addition or translocation lines. The alien chromosomes in the addition or translocation lines were not extensively intermixed with the wheat chromosomes and occupied discrete domains within the interphase nuclei (Heslop-Harrison et al. 1990; Mukai and Gill 1991).

Future outlook

Improvement of the resolution of the ISH technique

One of the most important future tasks will be to improve the resolution of the nonisotopic ISH technique in plants. At present, probes targeting DNA sequences shorter than 10 kb cannot be mapped by most laboratories. This will be the major limitation to wide application of ISH for plant genome mapping. The relatively low resolution of the nonisotopic ISH compared with that used in humans is probably due to the following factors: (*i*) the presence of cell walls and cytoplasmic debris; (*ii*) the more pronounced condensation of metaphase chromosomes; and (*iii*) lack of dedicated research on the optimization of the total hybridization process.

ISH using large genomic DNA clones as probes

The use of large genomic DNA clones can be considered as an alternative approach to map single-copy sequences in plants. In humans, cosmid or yeast artificial chromosome (YAC) clones can be directly mapped on metaphase chromosomes by ISH (Landegent et al. 1987; Lichter et al. 1990; Selleri et al. 1991). The cross-hybridization of the repetitive DNA sequences in the genomic clones can be suppressed through competition hybridization with Cot-1 DNA. This technique greatly facilitated the physical mapping of single-copy genes in humans. Genomic clones containing the target gene can be screened from the genomic libraries and then mapped by ISH.

With the present skills in several laboratories, unique DNA sequences of about 10 kb give detectable signals in ISH analysis. If repetitive DNA sequences account for 80% of plant genomic DNA, a 200-kb YAC or bacterial artificial chromosome (BAC, see Shizuya et al. 1992) clone theoretically should contain approximately 40 kb of unique sequences that will generate good ISH signals.

Interphase mapping

The order of two DNA probes separated by more than 1 Mb can be determined on metaphase chromosomes by ISH in humans (Lawrence et al. 1990). When the distance between two probes is less than 1 Mb, the ISH signals from these two probes result in a single spot on metaphase chromosomes. However, DNA sequences separated by 50 kb can be resolved in interphase nuclei (Lawrence et al. 1988; Trask 1991). Analysis of sequences separated from 50 kb to 1Mb apart indicated a strong correlation between interphase distance and linear DNA distance (Lawrence et al. 1990; Trask et al. 1989). With multicolor FISH analysis on interphase nuclei, Trask et al. (1992) determined the order of three probes spanning 300–500 kb.

Plant metaphase chromosomes are more condensed than human metaphase chromosomes. This is probably one of the reasons why low copy probe ISH is more difficult in plants. Thus interphase, the stage in which the chromosomes are decondensed, should be exploited for ISH mapping in plants. Exploiting the relationship between interphase and linear DNA distances in plants will be important for chromosome walking and map-based cloning. There is discrepancy between the genetic and physical distances in plant chromosomes (Werner et al. 1992). This is because the frequency of recombination is uneven along the length of a chromosome. Thus, the flanking DNA markers of a target gene sometimes cannot be mapped on a single DNA fragment in long range mapping, even though the markers are very close to each other on the genetic map. In these cases, interphase ISH mapping will be an alternative way to examine the physical distances between the flanking markers. The separation distance of the ISH sites in interphase can be used to estimate the linear DNA distance between the markers.

Cytogenetic determination of linkage relationship

Cytogenetic determination of the relationship between genetic linkage and individual chromosomes should be considered as one of the major future tasks of molecular cytogeneticists. Assigning a chromosome to a specific linkage group traditionally relies on aneuploid analysis, such as trisomic analysis. However, such aneuploid stocks are difficult to maintain and complete trisomic sets are not available for all crop plants. An efficient and valuable method for cytogenetic identification of the relationship between genetic linkage and individual chromosomes could be developed using ISH analysis. DNA sequences specific to a particular linkage group can be converted to ISH markers for cytogenetic identification. If the ISH technique using large genomic DNA clones as probes can be developed in plants, ISH markers can be screened from YAC and BAC libraries. Sequential banding and ISH analysis can be used to locate the ISH markers to specific chromosomes. ISH markers will also be very useful to analyze chromosome structure modification and evolution among related species.

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Ambros, P.F., Matzke, M.A., and Matzke, A.J.M. 1986. Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. Chromosoma, **94**: 11–18.

Anamthawat-Jónsson, K., and Heslop-Harrison, J.P. 1993. Isolation and characterization of genome-specific DNA sequences in *Triticeae* species. Mol. Gen. Genet. **240**: 151–158.

Anamthawat-Jónsson, K., Schwarzacher, T., and Heslop-Harrison, J.S. 1993. Behavior of parental genomes in the hybrid *Hordeum vulgare* × *H. bulbosum*. J. Hered. **84**: 78–82.

Appels, R., Morgan, L.B., and Gustafson, J.P. 1986. Rye heterochromatin. I. Studies on clusters of the major repeating sequences and the identification of a new dispersed repetitive sequence element. Can. J. Genet. Cytol. **28**: 645–657.

Appels, R., Dennis, E.S., Smyth, D.R., and Peacock, W.J. 1981. Two repeated DNA sequences from the heterochromatic regions of rye (*Secale cereale*) chromosomes. Chromosoma, **84**: 265–277.

Bedbrook, J.R., Jones, J., O'Dell, M., Thompson, R.D., and Flavell, R.B. 1980. A molecular description of telomeric heterochromatin in *Secale* species. Cell, **19**: 545–560.

Bennett, S.T., Kenton, A.Y., and Bennett, M.D. 1993. Genomic *in situ* hybridization reveals the allopolyploid nature of *Millium montianum* (Gramineae). Chromosoma, **101**: 420–424.

Bergey, D., Stelly, D.M., Price, H.J., and McKnight, T.D. 1989. *In situ* hybridization of biotinylated DNA probes to cotton meiotic chromosomes. Stain Technol. **64**: 25–37.

Chen, P.D., Tsujimoto, H., and Gill, B.S. 1994. Transfer of *Ph'* genes promoting homoeologous pairing from *Triticum speltoides* into common wheat and their utilization in alien genetic introgression. Theor. Appl. Genet. In press.

Chen, Q., and Armstrong, K.C. 1994. Genomic in situ hybridization in *Avena sativa*. Genome **37**. In press.

Clark, M., Karp, A., and Archer, S. 1979. Physical mapping of the B-hordein loci on barley chromosome 5 by *in situ* hybridization. Genome, **32**: 925–929.

Crane, C.F., Price, H.T., Stelly, D.M., and Czeschin, D.G., Jr. 1993. Identification of a homologous chromosome pair by in situ hybridization to ribosomal RNA loci in meiotic chromosomes of cotton (*Gossypium hirsutum*). Genome, **36**: 1015–1022.

Durnam, D.M., Gelinas, R., and Myerson, D. 1985. Detection of species specific chromosomes in somatic cell hybrids. Somatic Cell Mol. Genet. **11**: 571–577.

Friebe, B., Mukai, Y., Dhaliwal, H.S., Martin, T.J., and Gill, B.S. 1991. Identification of alien chromatin specifying resistance to wheat streak mosaic virus and greenbug in wheat germplasm by C-banding and *in situ* hybridization. Theor. Appl. Genet. **81**: 381–389.

Friebe, B., Zeller, F.J., Mukai, Y., Forster, B.P., Bartos, P., and McIntosh, R.A. 1992. Characterization of rust-resistant wheat – *Agropyron intermedium* derivatives by C-banding, *in situ* hybridization and isozyme analysis. Theor. Appl. Genet. **83**: 775–782.

Friebe, B., Gill, B.S., Mukai, Y., and Maan, S.S. 1993*a*. A noncompensating wheat–rye translocation maintained in perpetual monosomy in alloplasmic wheat. J. Hered. **84**: 126–129.

Friebe, B., Jiang, J., Gill, B.S., and Dyck, P.L. 1993*b*. Radiation-induced nonhomoeologous wheat – *Agropyron intermedium* chromosomal translocations conferring resistance to leaf rust. Theor. Appl. Genet. **86**: 141–149.

Friebe, B., Jiang, J., Knott, D.R., and Gill, B.S. 1994*a*. Compensation indices of radiation-induced wheat – *Agropyron elongatum* translocations conferring resistance to leaf rust and stem rust. Crop Sci. **34**: 400–404.

Friebe, B., Jiang, J., Raupp, W.J., and Gill, B.S. 1994*b*. Molecular cytogenetic analysis of radiation-induced alien genetic transfers in wheat. Proc. Int. Wheat Genet. Symp., 8th, 1994. In press.

Gall, J.G., and Pardue, M.L. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Natl. Acad. Sci. U.S.A. **63**: 378–383.

Griffor, M.C., Vodkin, L.O., Singh, R.J., and Hymowitz, T. 1991. Fluorescent *in situ* hybridization to soybean metaphase chromosomes. Plant Mol. Biol. **17**: 101–109.

Guidet, F., Rogowsky, P., Taylor, C., Song, W., and Langridge, P. 1991. Cloning and characterization of a new rye-specific repeated sequence. Genome, **34**: 81–87.

Gustafson, J.P., and Dillé, J.E. 1992. Chromosome location of *Oryza sativa* recombination linkage groups. Proc. Natl. Acad. Sci. U.S.A. **89**: 8646–8650.

Gustafson, J.P., Butler, E., and McIntyre, C.L. 1990. Physical mapping of a low-copy DNA sequence in rye (Secale *cereale* L.). Proc. Natl. Acad. Sci. U.S.A. **87**: 1899–1902.

Heslop-Harrison, J.S., Leitch, A.R., Schwarzacher, T., and Anamthawat-Jónsson, K. 1990. Detection and characterization of 1B/1R translocations in hexaploid wheat. Heredity, **65**: 385–392.

Hong, K.S., Richter, T.E., Bennetzen, J.L., and Hulbert, S.H. 1993. Complex duplications in maize lines. Mol. Gen. Genet. **239**: 115–121.

Hutchinson, J., and Seal, A.G. 1983. A sequential *in situ* hybridization and C-banding technique. Heredity, **51**: 507–509.

Jellen, E.N., Gill, B.S., and Cox, T.S. 1994. Genomic in situ hybridization differentiates between A/D- and C-genome chromatin and detects intergenomic translocations in polyploid oat species (genus *Avena*). Genome, **37**. In press.

Jiang, J., and Gill, B.S. 1993*a*. Sequential chromosome banding and in situ hybridization analysis. Genome, **36**: 792–795.

Jiang, J., and Gill, B.S. 1993*b*. A 'zebra' chromosome arising from multiple translocations involving nonhomologous chromosomes. Chromosoma, **102**: 612–617.

Jiang, J., and Gill, B.S. 1994*a*. Different species-specific chromosome translocations in *Triticum timopheevii* and *T. turgidum* supports diphyletic origin of polyploid wheats. Chromosome Res. **2**: 59–64.

Jiang, J., and Gill, B.S. 1994*b*. New 18S·26S ribosomal RNA gene loci: chromosomal landmarks for the evolution of polyploid wheat. Chromosoma, **103**: 179–185.

Jiang, J., Friebe, B., Dhaliwal, H.S., Martin, T.J., and Gill, B.S. 1993. Molecular cytogenetic analysis of *Agropyron elongatum* chromatin in wheat germplasm specifying resistance to wheat streak mosaic virus. Theor. Appl Genet. **86**: 41–48.

Jiang, J., Friebe, B., and Gill, B.S. 1994*a*. Recent advances in alien gene transfer in wheat. Euphytica, **73**: 199–212.

Jiang, J., Morris, K.L.D., and Gill, B.S. 1994*b*. Introgression of *Elymus trachycaulus* chromatin into common wheat. Chromosome Res. **2**: 3–13.

John, H.A., Birnstiel, M.L., and Jones, K.W. 1969. RNA–DNA hybrids at the cytological level. Nature (London), **223**: 582–587.

Kenton, A., Parokonny, A.S., Gleba, Y.Y., and Bennett, M.D. 1993. Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. Mol. Gen. Genet. **240**: 159–169.

Kim, N.-S., Armstrong, K., and Knott, D.R. 1993*a*. Molecular detection of *Lophopyrum* chromatin in wheat-*Lophopyrum* recombinants and their use in the physical mapping of chromosome 7D. Theor. Appl. Genet. **85**: 561–567.

Kim, N.-S., Armstrong, K.C., Fedak, G., Fominaya, A., and Whelan, E.W.P. 1993*b*. Cytological and molecular characterization of a chromosome interchange and addition lines in Cadet involving chromosome 5B of wheat and 6Ag of *Lophopyrum ponticum*. Theor. Appl. Genet. **86**: 827–832.

King, I.P., Purdie, K.A., Orford, S.E., Reader, S.M., and Miller, T.E. 1993. Detection of homoeologous chiasma formation in *Triticum durum* × *Thinopyrum bessarabicum* hybrids using genomic *in situ* hybridization. Heredity, **71**: 369–372.

Landegent, J.E., Jensen in de Wal, N., Dirks, R.W., Baas, F., and van der Ploeg, M. 1987. Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive *in situ* hybridization. Hum. Genet. **77**: 366–370.

Langer-Safer, P.R., Levine, M., and Ward, D.C. 1982. Immunological method for mapping genes on *Drosophila* polytene chromosomes. Proc. Natl. Acad. Sci. U.S.A. **79**: 4381–4385.

Lapitan, N.L.V., Sears, R.G., Rayburn, A.L., and Gill, B.S. 1986. Wheat–rye translocations. Detection of chromosome breakpoints by *in situ* hybridization with a biotin-labeled DNA probe. J. Hered. **77**: 415–419.

Lapitan, N.L.V., Gill, B.S., and Sears, R.G. 1987. Genomic and phylogenetic relationships among rye and perennial species in the Triticeae. Crop Sci. **27**: 682–686.

Lapitan, N.L.V., Ganal, M.W., and Tanksley, S.D. 1989. Somatic chromosome karyotype of tomato based on *in situ* hybridization of the TAG1 satellite repeat. Genome, **32**: 992–998.

Lapitan, N.L.V., Ganal, M.W., and Tanksley, S.D. 1991. Organization of the 5S ribosomal RNA genes in the genome of tomato. Genome, **34**: 509–514.

Lawrence, J.B., Villnace, C.A., and Singer, R.H. 1988. Sensitive, high-resolution chromatin and chromosome mapping *in situ*: presence and orientation of two closely intergrated copies of EBV in a lymphoma line. Cell, **52**: 51–61.

Lawrence, J.B., Singer, R.H., and McNeil, J.A. 1990. Interphase and metaphase resolution of different distance within the human dystrophia gene. Science (Washington, D.C.), **249**: 928–932.

Le, H.T., and Armstrong, K.C. 1991. In situ hybridization as a rapid means to assess meiotic pairing and detection of alien DNA transfers in interphase cells of wide crosses involving wheat and rye. Mol. Gen. Genet. **225**: 33–37.

Le, H.T., Armstrong, K.C., and Miki, B. 1989. Detection of rye DNA in wheat–rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Mol. Biol. Rep. **7**: 150–158.

Lehfer, H., Busch, W., Martin, R., and Herrmann, R.G. 1993. Localization of the B-hordein locus on barley chromosomes using fluorescence *in situ* hybridization. Chromosoma, **102**: 428–432.

Leitch, A.R., Mosgöller, W., Schwarzacher, T., Bennett, M.D., and Heslop-Harrison, J.S. 1990. Genomic *in situ* hybridization to sectioned nuclei shows chromosome domains in grass hybrids. J. Cell. Sci. **95**: 335–341.

Leitch, A.R., Schwarzacher, T., Mosgöller, W., Bennett, M.D., and Heslop-Harrison, J.S. 1991. Parental genomes are separated throughout the cell cycle in a plant hybrid. Chromosoma, **101**: 206–213.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by *in situ* hybridization. Genome, **35**: 1013–1018.

Leitch, I.J., and Heslop-Harrison, J.S. 1993. Physical mapping of four sites of 5S rDNA sequences and one site of the α-amylase-2 gene in barley (*Hordeum vulgare*). Genome, **36**: 517–523.

Leitch, I.J., Leitch, A.R., and Heslop-Harrison, J.S. 1991. Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. Genome, **34**: 329–333.

Lichter, P., Tang, C.C., Call, K., Hermanson, G., Evans, G.A., Housman, D., and Ward, D.C. 1990. High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science (Washington, D.C.), **247**: 64–69.

Liu, C.J., Atkinson, M.D., Chinoy, C.N., Devos, K.M., and Gale, M.D. 1992. Nonhomoeologous translocations between group 4, 5 and 7 chromosomes within wheat and rye. Theor. Appl. Genet. **83**: 305–312.

Maluszynska, J., and Heslop-Harrison, J.S. 1991. Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. The Plant J. **1**: 159–166.

Manuelidis, L., Langer-Safer, P.R., and Ward, D.C. 1982. High-resolution mapping of satellite DNA using biotin-labeled DNA probes. J. Cell Biol. **95**: 619–625.

McNeil, J.A., Johnson, C.V., Carter, K.C., Singer, R.H., and Lawrence, J.B. 1991. Localizing DNA and RNA within nuclei and chromosomes by fluorescence *in situ* hybridization. Genet. Anal. Tech. Appl. **8**: 41–58.

Mukai, Y., and Gill, B.S. 1991. Detection of barley chromatin added to wheat by genomic *in situ* hybridization. Genome, **34**: 448–452.

Mukai, Y., Endo, T.R., and Gill, B.S. 1990. Physical mapping of the 5S rRNA multigene family in common wheat. J. Hered. **81**: 290–295.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping of the 18S·26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.

Mukai, Y., Friebe, B., and Gill, B.S. 1992. Comparison of C-banding patterns and *in situ* hybridization sites using highly repetitive and total genomic rye DNA probes of 'Imperial' rye chromosomes added to 'Chinese Spring' wheat. Jpn. J. Genet. **67**: 71–83.

Mukai, Y., Nakahara, Y., and Yamamoto, M. 1993*a*. Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence *in situ* hybridization using total genomic and highly repeated DNA probes. Genome, **36**: 489–494.

Mukai, Y., Friebe, B., Hatchett, J.H., Yamamoto, M., and Gill, B.S. 1993*b*. Molecular cytogenetic analysis of radiation-induced wheat–rye terminal and intercalary chromosomal translocations and the detection of rye chromatin specifying resistance to Hessian fly. Chromosoma, **102**: 88–95.

Naranjo, T. 1990. Chromosome structure of durum wheat. Theor. Appl. Genet. **79**: 397–400.

Naranjo, T., Roca, A., Goicoecha, P.G., and Giraldez, R. 1987. Arm homoeology of wheat and rye chromosomes. Genome, **29**: 873–882.

Okamoto, M., 1962. Identification of the chromosomes of common wheat belonging to the A and B genomes. Can. J. Genet. Cytol. **4**: 31–37.

Pinkel, D., Straume, T., and Gray, J.W. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. U.S.A. **83**: 2934–2938.

Rajaram, S., Mann, C.H.E., Ortiz-Ferrara, G., and Mujeeb-Kazi, A. 1983. Adaptation, stability and high yield potential of certain 1B/1R CIMMYT wheat. Proc. Int. Wheat Genet. Symp., 6th, 1983. pp. 613–621.

Rayburn, A.L., and Gill, B.S. 1985. Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. J. Hered. **76**: 78–81.

Rayburn, A.L., and Gill, B.S. 1986. Molecular identification of the D-genome chromosomes of wheat. J. Hered. **77**: 253–255.

Rayburn, A.L., and Gill, B.S. 1988. Repeated DNA sequences in *Triticum* (*Poaceae*): chromosomal mapping and its bearing on the evolution of B and G genomes. Plant Syst. Evol. **159**: 229–235.

Ricroch, A., Peffley, E.B., and Baker, R.J. 1992. Chromosomal location of rDNA in *Allium*: *in situ* hybridization using biotin- and fluorescein-labelled probe. Theor. Appl. Genet. **83**: 413–418.

Schaff, D.A., Koehler, S.M., Matthews, B.F., and Bauchan, G.R. 1990. *In situ* hybridization of β-Tubulin to alfalfa chromosomes. J. Hered. **81**: 480–483.

Schwarzacher, T., Leitch, A.R., Bennett, M.D., and Heslop-Harrison, J.S. 1989. In-situ localization of parental genomes in a wide hybrid. Ann. Bot. (London), **64**: 315–324.

Sears, E.R. 1954. The aneuploids of common wheat. Mo. Agric. Exp. Stn. Bull. No. 572.

Selleri, L., Hermanson, G., Eubanks, J.H., and Evans, G.A. 1991. Chromosomal *in situ* hybridization using yeast artificial chromosomes. Genet. Anal. Tech. Appl. **8**: 59–66.

Shizuya, H., Birren, B., Kim, U.J., Mancina, V., Splepak, T., and Tachiiri, Y. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. U.S.A. **89**: 8794–8797.

Simpson, P.R., Newman, M.-A., and Davies, D.R. 1988. Detection of legumin gene DNA sequences in pea by *in situ* hybridization. Chromosoma, **96**: 454–458.

Skorupska, H., Albertsen, M.C., Langholz, K.D., and Palmer, R.D. 1989. Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by *in situ* hybridization. Genome, **32**: 1091–1095.

The, T.T., Gupta, R.B., Dyck, P.L., Appels, R., Hohmann, U., and McIntosh, R.A. 1992. Characterization of stem rust resistant derivatives of wheat cultivar Amigo. Euphytica, **58**: 245–252.

Trask, B.J. 1991. Gene mapping by *in situ* hybridization. Curr. Opin. Genet. Dev. **1**: 82–87.

Trask, B.J., Pinkel, D., and van den Engh, G. 1989. The proximity

of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmid spanning 250 kilobase pairs. Genomics, **5**: 710–717.

Trask, B.J., Massa, H.F., and Burmeister, M. 1992. Fluorescence *in situ* hybridization establishes the order cen-DXS28(C7)-DXS67(B24)-DXS68(L1)-tel in human chromosome Xp21.3. Genomics, **13**: 455–457.

Tsujimoto, H., and Gill, B.S. 1991. Repetitive DNA sequences from polyploid *Elymus trachycaulus* and the diploid progenitor species — detection and genomic affinity of *Elymus* chromatin added to wheat. Genome, **34**: 782–789.

Tsunewaki, K., and Ogihara, Y. 1983. The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops* species. II. On the origin of polyploid wheat cytoplasms as suggested by chloroplast DNA restriction fragment patterns. Genetics, **104**: 155–171.

Viegas-Péquignot, E., Berrard, S., Brice, A., Apiou, F., and Mallet, J. 1991. Localization of a 900-bp-long fragment of the human choline acetyltransferase gene to 10q11.2 by non-radioactive *in situ* hybridization. Genomics, **9**: 210–212.

Werner, J.E., Endo, T.R., and Gill, B.S. 1992. Toward a cytogenetically based physical map of the wheat genome. Proc. Natl. Acad. Sci. U.S.A. **89**: 11307–11311.

Fluorescent in situ hybridization and C-banding analyses of highly repetitive DNA sequences in the heterochromatin of rye (*Secale montanum* Guss.) and wheat incorporating *S. montanum* chromosome segments

Angeles Cuadrado and Nicolás Jouve

Abstract: The molecular characterization of C-banded regions of *Secale montanum* Guss. by means of in situ hybridization was performed in order to provide new information about their chromosome structure relative to cultivated rye, *Secale cereale* L. Accurate identification of individual chromosomes was achieved using simultaneous and (or) successive fluorescent in situ hybridization (FISH) and C-banding. FISH identification was performed using total rye DNA, three highly repetitive rye DNA sequences (pSc119.2, pSc74, and pSc34), and the ribosomal RNA probes pTa71 (18S, 5.8S, and 26S rDNA) and pTa794 (5S rDNA). FISH was also used to identify the chromosome segment involved in two spontaneous translocation lines recovered from a 'Chinese Spring' – *S. montanum* wheat–rye addition line. FISH analysis revealed the exact translocation breakpoints and allowed the identification of the transferred rye segments. The value of this type of analysis is discussed.

Key words: *Secale cereale*, *Secale montanum*, rye, repetitive DNA, fluorescence in situ hybridization.

Résumé : L'hybridation in situ par fluorescence (FISH) a été employée pour caractériser, au niveau moléculaire, les régions à bandes C du *Secale montanum* Guss. afin de mieux définir la structure des chromosomes de celui-ci par rapport à ceux du seigle cultivé, *Secale cereale*. Une identification précise des chromosomes individuels a été réalisée en employant simultanément ou successivement l'hybridation in situ et le marquage des bandes C. L'identification par marquage FISH a été réalisée en utilisant comme sonde l'ADN total de seigle, trois séquences hautement répétées chez le seigle (pSc119.2, pSc74 et pSc34) et les sondes d'ADN ribosomique pTa71 (ADNr 18S, 5.8S et 26S) et pTa794 (ADNr 5S). La technique FISH a également été employée afin d'identifier un segment de chromosome impliqué dans deux translocations spontanées dérivées d'une lignée d'addition blé–seigle ('Chinese Spring' – *S. montanum*). L'analyse FISH a permis de localiser précisément le point de translocation et d'identifier les segments transférés du seigle. L'utilité de ce type d'analyse est discutée.

Mots clés : *Secale cereale*, *Secale montanum*, seigle, ADN répété, hybridation in situ par fluorescence.
[Traduit par la Rédaction]

Introduction

The use of in situ hybridization in conjunction with biotin-labelled probes allows the identification of individual chromosomes in plant species by the physical mapping of defined sequences. The first use of this technique for mapping plant chromosomes was made in wheat using a repeated DNA sequence from rye (Rayburn and Gill 1985). Since then, fluorescent in situ hybridization (FISH) analysis using biotin-labelled DNA probes with repeated sequences has

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A. Cuadrado and N. Jouve.[1] Department of Cell Biology and Genetics, University of Alcalá de Henares, E-28871, Alcalá de Henares, Madrid, Spain.

[1] Author to whom all correspondence should be addressed.

been widely used for chromosome and genome identification in wheat and its relatives (Rayburn and Gill 1987; Lapitan et al. 1987; McIntyre et al. 1990; Mukai et al. 1990, 1991, 1993*b*; Friebe et al. 1991).

Since a probe can be labelled with a variety of different haptens, it is now possible to detect two or more DNA sequences in plant chromosomes using mixed FISH (Leitch et al. 1991). In this way Mukai et al. (1993*b*) identified the somatic chromosomes of the wheat *Triticum aestivum*. A similar characterization has been recently carried out by Cuadrado and Jouve (1994) in rye chromosomes of hexaploid triticale.

The localization and identification of translocated chromosome segments introduced into wheat from other species is a further use of FISH techniques. Here, it is necessary to use probes that give species-specific hybridization patterns. It is possible to identify alien chromatin, introduced by crossing or other methods, using specific DNA sequences or total genomic DNA from the donor partner as probes (Lapitan et al. 1986; Rayburn and Gill 1987; Le et al. 1989; Heslop-Harrison et al. 1990; Mukai and Gill 1991; Guidet et al. 1991; Schwarzacher et al. 1992; Mukai et al. 1993*a*).

In this paper, in situ hybridization of *Secale montanum* chromosomes with three different highly repetitive DNA sequences from *Secale cereale* is discussed. The characterization of the chromosomes of *S. montanum* by their in situ patterns provides a new and useful way to compare the karyotypes of rye and other species. It also provides a new means of identifying chromosome regions involved in several spontaneous translocations found in addition lines of *T. aestivum* cv. Chinese Spring with introduced *S. montanum* chromosomes.

Materials and methods

Plant materials

The plant material used in this investigation included one sample of *S. montanum* Guss. ($2n = 14$; genome R^mR^m) from the working collection of the Genetics Unit of the University of Alcalá de Henares and segregant specimens of *T. aestivum* L. cv. Chinese Spring ($2n = 6x = 42$; genomes AABBDD) with two structural terminal translocations from *S. montanum* of different lengths on the 5BS arm of wheat. The 5B-translocated segments from *S. montanum* appeared spontaneously in the progeny of the *T. aestivum* cv. Chinese Spring telocentric $5R^m$ addition line.

DNA probes

Genomic DNA was isolated from leaves of the rye *S. cereale* L. cv. Petkus. The probes employed in FISH analyses included pSc119.2, pSc74, and pSc34 containing, respectively, the highly repeated DNA sequences of 120, 350–480, and 610 base pairs (bp) derived from *S. cereale* (Bedbrook et al. 1980; Jones and Flavell 1982*a*; McIntyre et al. 1990). Two different rDNA probes were also used, pTa71, containing the 18S, 5.8S, and 26S ribosomal sequences, and pTa794, containing the 5S ribosomal sequence (Gerlach and Bedbrook 1979; Gerlach and Dyer 1980).

Chromosome preparation, FISH, and C-banding

Chromosomes were prepared by squashing enzyme-softened root tips following pretreatment in ice water and fixation in ethanol (99%) – glacial acetic acid (3:1). After removal of the cover slip and subsequent RNase treatment, slides were fixed in 4% (w/v) paraformaldehyde, dehydrated in a graded ethanol series, and air-dried. The in situ hybridization was carried out using standard methods with a hybridization stringency of 85%.

The plasmid clones pSc34, pSc119.2, and pTa794 were amplified and labelled with digoxigenin-11-dUTP (Boehringer Mannheim) using the polymerase chain reaction (PCR). Clone pSc74 was labelled with rhodamine and amplified using PCR. Finally, the plasmid clone pTa71 and rye total DNA were labelled with rhodamine-4-dUTP (Amersham) by nick translation using the BioNick Labelling System (Gibco BRL). Chromosome and probe denaturation and in situ hybridization steps were carried out as described by Heslop-Harrison et al. (1991) and, more recently, Cuadrado and Jouve (1994).

Chromosome preparations were denatured prior to in situ hybridization using a programmable thermal cycler (PT-100, M.-J. Research Inc.). Simultaneous hybridization (with the probes added just prior to use) and reprobing were then carried out. After the last hybridization process, the cover slip was removed and the slides were immersed in ethanol (99%) – glacial acetic acid (3:1) for 60 min. The slides were then dehydrated in absolute alcohol for about 60 min and air-dried overnight, following the C-banding technique of Lukaszewski and Gustafson (1983).

Results and discussion

Karyotyping of *S. montanum* chromosomes by FISH

The different DNA probes labelled each *S. montanum* chromosome differently. The in situ hybridization sites and corresponding C-banding pattern of each chromosome are given in Fig. 1. The C-banding patterns of chromosomes in various samples of *S. montanum* were previously reported by Gustafson et al. (1976), who observed that banding of all seven chromosomes was fairly constant from one specimen to another. Further, these chromosomes were easily identifiable and could be distinguished by C-banding pattern from the seven *S. cereale* chromosomes.

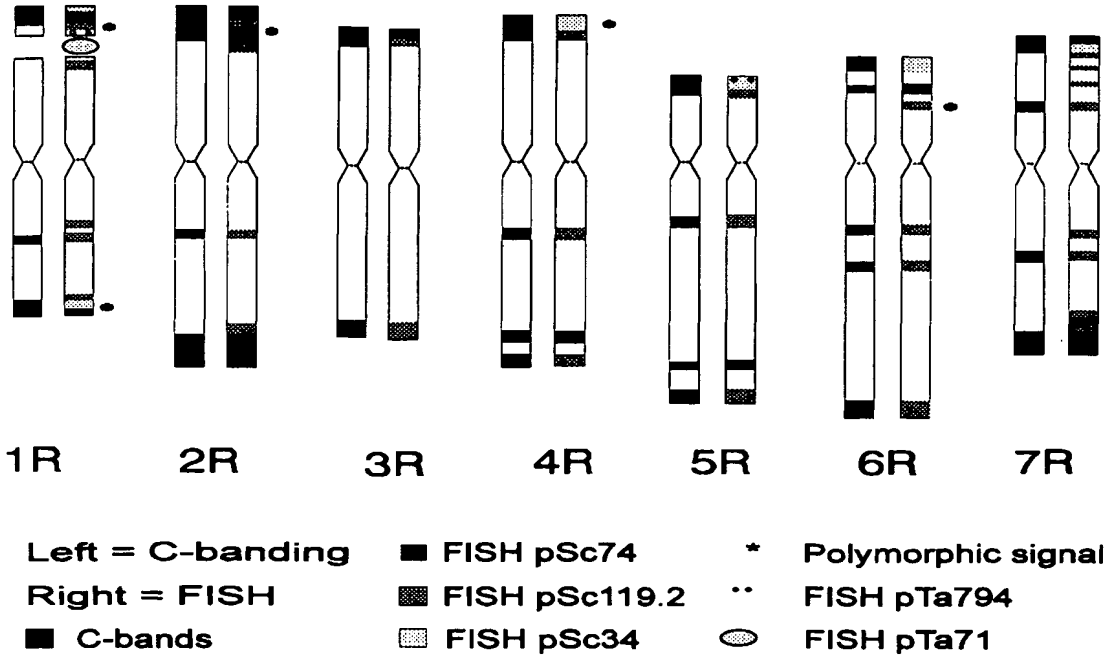
In the discussion of the individual *S. montanum* chromosomes that follows, in situ hybridization and C-banding patterns are considered together and compared with the C-banding results of Gustafson et al. (1976) for *S. montanum* and our own results for different *S. cereale* lines (Cuadrado and Jouve 1994; A. Cuadrado, unpublished data). The chromosome designations of Gustafson et al. (1976) are given in single quotes in parentheses.

Chromosome $1R^m$ ('A')

This is a submetacentric chromosome carrying a satellite chromosome with a moderately weak terminal block of heterochromatin on its long arm. A small C-band occupies a position halfway along the length of the long arm and the short arm presents a prominent terminal band adjacent to the satellite (Fig. 2*j*). The telomeric heterochromatin shows some variation in size between homologous chromosomes within and between different plants.

Both telomeres show a complex organization formed by two or more repetitive sequences. There are a maximum

Fig. 1. The physical maps of *S. montanum*: C-banding (left) and FISH to 120, 480, and 610 bp DNA repetitive families of rye (right).



of five hybridization sites with the probe pSc119.2: the first at the proximal end of the satellite, the second near the NOR region, the third and fourth variably present at interstitial sites on the long arm, and the fifth at the distal end of the long arm. Probes pSc74 and pSc34 hybridize to both telomeres of 1R. The 5S rDNA locus was found near the satellite, close to the NOR region, after hybridization with the probe pTa794 (Fig. 2*c*). Finally, the probe pTa71 hybridizes with the NOR site. The hybridization pattern of 1R of *S. montanum* is very similar to that of *S. cereale* with only minor quantitative differences in the pSc74 site.

Chromosome 2R^m ('G')

This is the largest chromosome of the karyotype. It is metacentric to submetacentric and has obvious terminal bands on both arms. The long arm generally shows a band occupying a proximal position $^1/_3$–$^1/_2$ the distance towards the end of the arm. pSc119.2 is present in both telomeres. It hybridizes weakly at a site close to the large telomeric site of pSc74 and at another site distal to, or within, the telomeric block of pSc74 in both telomeres (Figs. 2*e* and 2*i*). Variation in the distribution of the 480 bp DNA repetitive sequence was detected between homologous chromosomes of the same cell. An intercalary hybridization site of pSc119.2 is observed occupying the same position as the interstitial C-band. Occasionally pSc34 hybridizes to the telomere 2R^{m}S (Fig. 2*b*).

The in situ hybridization results suggest that chromosome 2R^m of *S. montanum* corresponds to the chromosome designated 'G' by Gustafson et al. (1976) and not 'B', as was suggested by these authors. The possible misclassification of this chromosome is inferred from two pieces of evidence: (*i*) though both chromosomes are almost metacentric, 'G' is bigger than 'B' (as 2R is bigger than 4R/7R) and (*ii*) the pattern of repetitive DNA of 2R in this investigation matches the C-banding pattern of 'G' better than it does that of 'B'. This is in agreement with the observed distribution of the hybridization sites of pSc119.2

Chromosome 3R^m ('C)

This is a metacentric to submetacentric chromosome that shows thin heterochromatic bands in both telomeres. It only shows telomeric hybridization sites for the probes pSc119.2 (both arms) and pSc74 (a weak signal at the proximal telomeric position in the short arm). The probe pSc34 does not hybridize to this chromosome (Figs. 2*h*–2*j*).

Chromosome 4R^m ('D')

This is a submetacentric chromosome with a characteristic terminal band in the short arm. The long arm consists of two weak interstitial bands with the most characteristic band being located approximately $^1/_3$–$^1/_2$ the length of the arm away from the centromere. The probe pSc74 hybridizes at a major interstitial site near the telomere of the long arm. The probe pSc34 exhibits polymorphic hybridization in the telomere of the short arm (Figs. 2*b* and 2*j*). On the other hand, pSc119.2 shows two readily visible sites in the long arm in the same positions as C-bands. The first of these two sites is terminal and the other is interstitial lying $^1/_3$ the length of the arm away from the centromere.

The hybridization and C-banding patterns of chromosome 4R of *S. montanum* are very similar to those exhibited by its counterpart in *S. cereale*. The site of hybridization with pSc74, close to the telomere in 4R^{m}L, corresponds well to the position of the interstitial C-band found in both species (Figs. 2*a* and 2*h*).

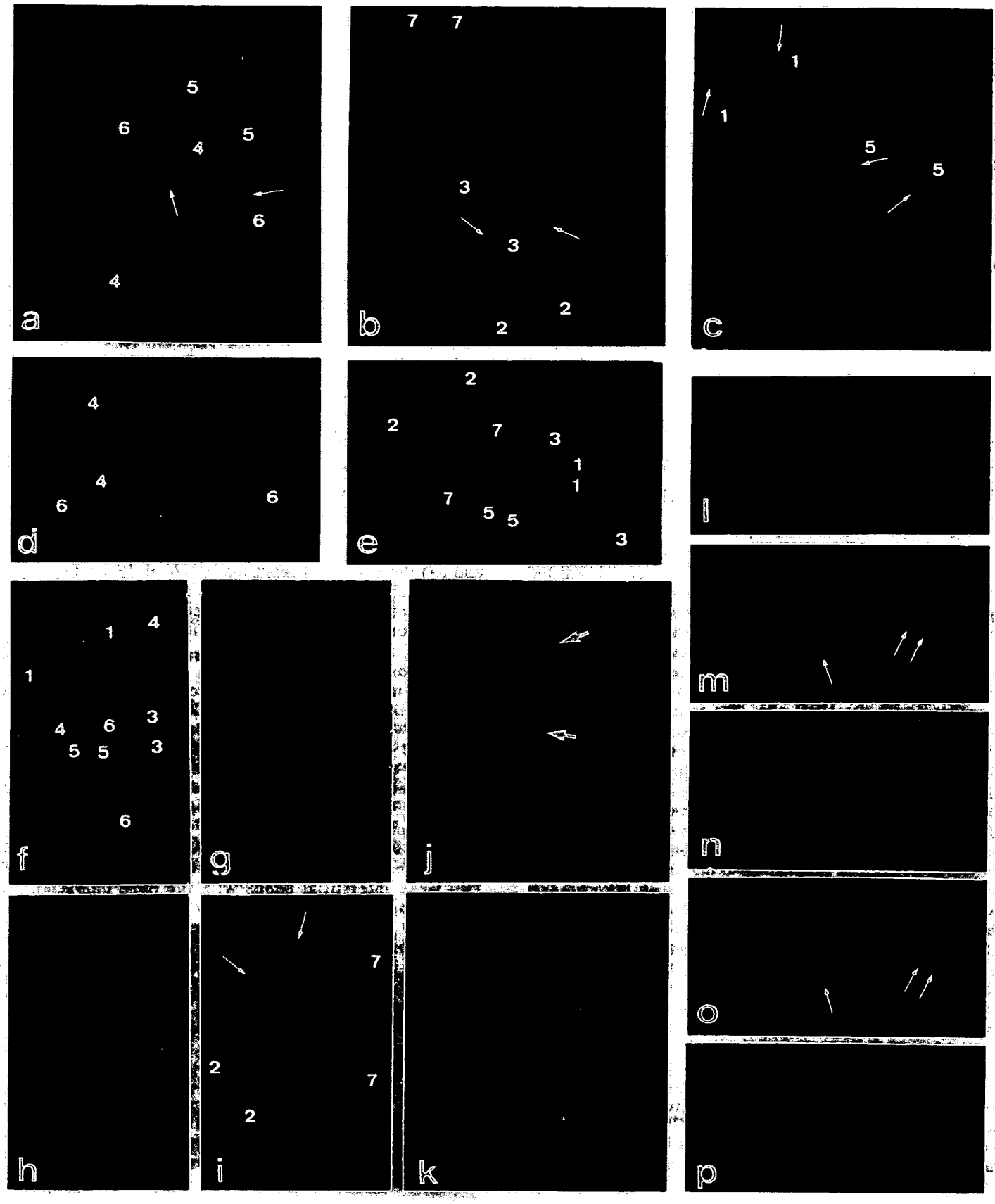

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Fig. 2. Multicolor results of the in situ hybridization to localize the sites for different DNA probes. (*a–i*) Metaphase plates from *S. montanum*. (*a*, *b*, *c*) The same cell after hybridization to pSc74 (*a*), pSc34 (*b*), and pTa794 (*c*). Note the variation in the intensity of the signal between homologues in the telomeres of 2RS. In *a* the arrows show the sites for the pSc74 in 6RS, in *b* they show the polymorphic telomere 4RS, and in *c* they show the rDNA 5S sites in 1R and 5R. (*d*, *e*) The same cell counterstained with DAPI (*e*), and showing the sites for the probe pSc119.2 (green) (*d*). (*f–i*) The same metaphase showing DAPI counterstaining (*f*) and the hybridization sites for probes pSc74 (red) (*g*), pSc119.2 (green) (*g*), pSc34 (red) (*h*), pSc119.2 (green) (*h*), and pSc34 (red) against DAPI (*i*). The arrows in *i* show the polymorphism for the telomere 1RS. (*j–p*) Genomic FISH to detect the chromatin of *S. montanum* in the wheat background of the translocated line *T. aestivum* – *S. montanum*, in interphase (*j*,*k*) and metaphase (*l–p*) cells. (*l–p*) A partial metaphase showing both types of grafted chromosomes present in a heterozygous plant. The photographs show simultaneous visualization of hybridization to rhodamine-labelled total DNA from rye against DAPI (*l*), FISH using pSc119.2 (*m*), FISH using pSc74 and pTa794 simultaneously (*n*), FISH using pTa794 (*o*), and DAPI (*p*). The reddish to red colours correspond to rhodamine-labelled probes. The yellow–green colours correspond to digoxigenin-labelled probes. The single and double arrows indicate, respectively, the single and duplicated signals on the primary and secondary grafted segments.

Chromosome 5R^m ('E')

This is the most subterminal of the seven chromosomes. It has a terminal C-band in the short arm. The long arm contains three characteristic C-bands occupying positions that are 1/4 the length of the arm away from the centromere, interstitial, and telomeric. This chromosome shows in situ hybridization with the probes pSc119.2 and pSc74 and has a distribution pattern similar to that of chromosome 4R (Fig. 2*e*). However, both chromosomes can be distinguished, since they have different morphology. Probe pSc34 hybridizes at a distal position in the short arm (Figs. 2*b* and 2*j*). Probe pTa794 hybridizes at a subtelomeric site in the short arm at the same position as in *S. cereale* (Fig. 2*c*).

Chromosome 6R^m ('F')

This is a submetacentric chromosome with a terminal C-band on the short arm and a series of interstitial bands on the long arm. The pSc74 probe hybridizes at a major interstitial site near the telomere of the short arm and at a subtelomeric site on the long arm that shows variations in size between homologous chromosomes within and between different plants (Figs. 2*a* and 2*h*). Probe pSc34 hybridizes at a telomeric position in the short arm. The major site is found in the distal end of the long arm. The 120 bp sequence detected by pSc119.2 is clustered at 3–4 hybridization sites in 6R^{m}L. The same arm shows a further two interstitial sites 1/4 and 1/3 the length of the arm away from the centromere. A minor polymorphic interstitial site is seen in the middle of the short arm (Figs. 2*e* and 2*i*). This chromosome is very similar in external morphology to 6R of *S. cereale*. The main difference between these species is the interstitial bands. Chromosome 6R^m shows a subtelomeric hybridization site for the 480 bp repetitive family in the short arm. However, this is found in the long arm in cultivated rye. The results obtained with this probe agree with the existence of a pericentromeric inversion that differentiates 6R^m and 6R (Devos et al. 1993).

Chromosome 7R^m ('B')

This is a metacentric to submetacentric chromosome. It presents a prominent terminal heterochromatic block on the long arm and a less important C-band in the telomere of the short arm. It occasionally shows a thin band halfway along the length of the short arm. A major telomeric hybridization site and three interstitial sites for the probe pSc119.2 were observed on the short arm. Moreover, it also hybridizes forming two doublets, the first at a position 1/2–1/3 of the way towards the end of the long arm and the second at a subtelomeric position in the same arm. The repetitive family of 480 bp is found in both telomeres distal to the 120 bp repetitive family. The hybridization region for pSc74 is larger in the telomere of the long arm than in the short arm. A subtelomeric hybridization site for pSc74 is seen to lie within the subtelomeric doublet of pSc119.2 in the long arm. The probe pSc34 hybridizes with the telomere of the short arm between the sites of the 120 bp and 480 bp repetitive family of sequences (Figs. 2*b* and 2*j*).

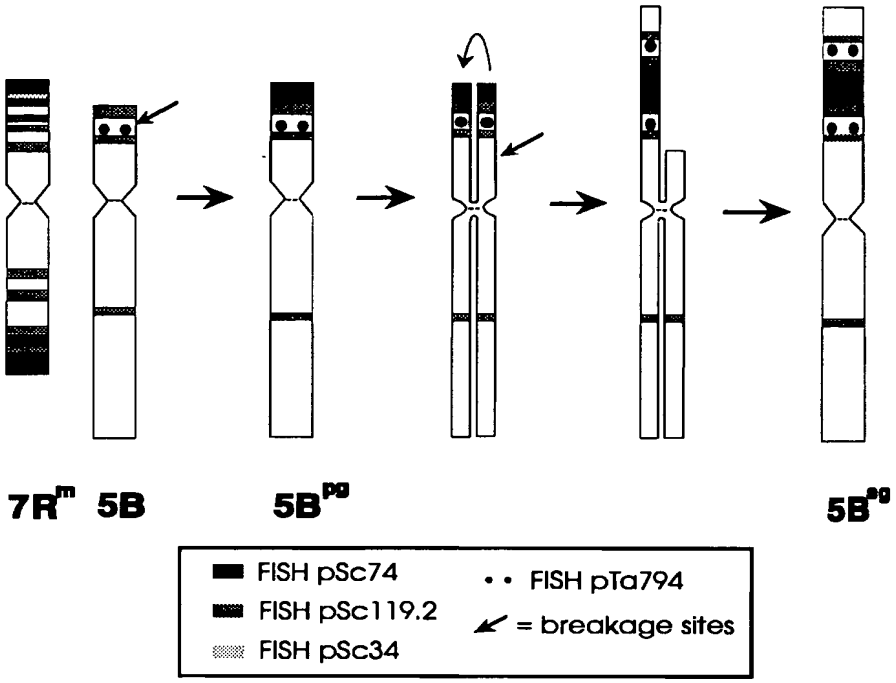
The short arm of chromosome 7R^m has a similar hybridization pattern to that of 7R of *S. cereale*. However, the long arms are clearly different owing to the large telomeric hybridization region observed in 7R^m.

Comparison of the FISH karyotypes of *S. cereale* and *S. montanum*

The simultaneous use of multiple targets in a single hybridization experiment permits the physical mapping of various DNA sequences along chromosomes. Using a combination of two different repetitive DNA sequences, it is possible to identify all chromosomes of both species. The results demonstrate that the physical locations of the repetitive DNA families of 120, 480, and 610 bp are rather similar to those of the chromosome of *S. montanum* and *S. cereale*. Most of the differences are quantitative rather than qualitative; differences in intensity in hybridization sites are usual within and between homologous chromosomes in open pollinated rye lines (N. Jouve, unpublished data). The main qualitative difference is the location of the 610 and 480 bp families: these are rarely combined in the same telomeres in *S. montanum*, a situation that is usually found in *S. cereale*.

Jones and Flavell (1982*b*) reported that both species have the same complement of repetitive DNA sequences but that they differed significantly in the amount of telomeric 610 bp repetitive sequences and in the pattern of distribution of the 480 bp repetitive sequences along the chromosomes. The results of this investigation demonstrate a similar distribution for the 120 bp repetitive family in most of the

Fig. 3. A scheme showing the possible origin of the grafted regions of chromosome $7R^m$ of *S. montanum* as putative donor in the line *T. aestivum* cv. Chinese Spring. $5B^{pg}$ is the primary grafted segment on chromosome 5B. $5B^{sg}$ is the secondary grafted segment on chromosome 5B.



telomeric and interstitial sites in chromosomes of both species. Differences in the distribution of hybridization sites for the pSc119.2 probe in both species were limited to chromosome arms 2RS, 2RL, and 7RL.

The 610 bp repetitive sequence hybridized exclusively to the telomeres, predominantly to the telomeric regions of chromosome arms 1RS, 1RL, 4RS, 5RS, 6RS, and 7RS. However, this sequence was not always seen in the 1RS arm of either species. It was almost always absent in the most prominent heterochromatic blocks of telomeres 2RS and was always absent in 2RL and 7RL in *S. montanum*. Jones and Flavell (1982*b*) reported a major difference in the hybridization responses of these species to the probe pSc34 (610 bp), hybridization being clearly more intense in *S. cereale*. This agrees well with the results of this investigation. However, no generalization can be made, since very weak in situ hybridization with the probe pSc34 was seen in other rye lines.

Finally, the use of the probe pSc74 to detect the distribution of the 480-bp sequence demonstrated its presence in almost all arms. Interestingly, interstitial hybridization sites are found in the short arm of 6R in *S. montanum* and in the long arm in *S. cereale* (Jones and Flavell 1982*a*; Lapitan et al. 1987; Mukai et al. 1992; Cuadrado and Jouve 1994). Thus, the family sequence of 480 bp hybridizes in a subtelomeric region of the short arm in *S. montanum* and in a position $^1/_2$–$^1/_3$ the length of the long arm distal to the centromere in *S. cereale*. Moreover, there are other interstitial hybridization sites for the probe pSc119.2. These occur between the positions of pSc74 and the centromere of 6RS (1 site) and between the positions of pSc74 and the centromere of 6RL (two sites) in both species. Other important differences were found when comparing the distribution of interstitial hybridization sites in chromosome arms 2RL and 7RL.

Translocations are thought to have played an important role in the evolution of the genus *Secale* (Stutz 1972). Van Heemert and Sybenga (1972) reported that the genomes of *S. cereale* and *S. montanum* differed by at least two interchanges involving chromosomes 2R, 6R, and 7R. This is in agreement with observations made of the same chromosomes in this work. The most important differences lie in the in situ patterns produced by probes pSc119.2, pSc74, and pSc34. Thus, 2RS, 2RL, 6RS, 6RL, and 7RS must have played an important role in these interchanges.

Shewry et al. (1985) located *Sec2* genes on chromosome $6R^m$ of *S. montanum* and concluded that there was a translocation between 2R and 6R in *S. cereale* relative to *S. montanum* (and probably to wheat, where homologous genes are located on 6AS, 6BS, and 6DS of wheat). This has been presented as evidence to explain a translocation between part of the short arm of a group 2 chromosome and part of the short arm of a group 6 chromosome (Devos et al. 1993). These results, which show an interstitial hybridization site for the probe pSc74 in $6R^m$ on opposite arms in the two species, support the possible occurrence of a pericentric inversion during the evolution of *S. montanum* to *S. cereale*, as suggested by Devos et al. (1993).

Introgression in *T. aestivum* cv. Chinese Spring – *S. montanum* addition lines

Montero et al. (1986) reported the existence of a spontaneous translocation in a 'Chinese Spring' – *S. montanum* wheat–rye telocentric 5R^{m}S addition line. The homozygous translocated plants that resulted had a level of pairing as low as that caused by the deficiency of 5BS. This led to the assumption that the terminal region containing the system that regulates pairing in 5BS had been deleted. The translocation appeared spontaneously in this aneuploid line and it was assumed that the terminal segment of 5B had been replaced by a short terminal region of a chromosome from *S. montanum*. Since this spontaneous chromosome aberration appeared in a sample of the 'Chinese Spring'–ditelo-5R^{m}S addition line, a terminal translocation from this arm in 5BS was thought to have occurred. C-banding did not facilitate the identification of telomeric regions of rye chromosomes, therefore neither the precise position of the breakpoint nor the *S. montanum* translocated region could be identified.

A method that identifies and explains the origin of the translocated region is required. Results from genomic FISH may provide the solution. During investigation of the chromosomal constitution of plants from the above mentioned line, many plants were seen to have lost the 5R^{m}S telocentric addition. Others conserved one or both added arms. Moreover, two domains of labelled chromatin were observed in interphase nuclei in these plants when using genomic in situ hybridization with interphase nuclei (Fig. 2*f*). This method is very convenient for screening and initial characterization of segregant plant material or recombinant lines that contain alien chromosome segments.

As well as identifying plants carrying translocated regions from *S. montanum*, attempts were made to identify the chromosome that donated the alien segment. Repetitive DNA sequences present in the translocated segment were studied in metaphase cells using different fluorescent labels for each DNA sequence. Surprisingly, the results show the presence of two chromosomal segments of different size from *S. montanum*. Both were grafted at distal position in chromosome arm 5BS and differed in size and in the distribution of their repetitive DNA.

Chromosome 5B can easily be identified by using its hybridization patterns for the DNA probes pSc119.2 and pTa794 as characterizing features (Rayburn and Gill 1985; Dvořák et al. 1989; Mukai et al. 1993*b*). The results of this investigation demonstrate that simultaneous and (or) successive FISH with the probes pSc119.2, pSc74, pSc34, and pTa794 and rye genomic DNA are also very useful in distinguishing the *S. montanum* chromosomes. Presumably the larger grafted segment came about as a result of a breakage in the chromosome, so that the first segment was retranslocated carrying an extra piece of chromatin. In the results discussed below, the original grafted segment is referred to as the primary segment and the retranslocated larger segment is referred to as the secondary segment.

Primary grafted segment

The alien segment from *S. montanum* was found in a distal position of the short arm of the wheat chromosome 5B. The short arm of the translocated chromosome exhibits: (*i*) wheat chromatin, including one proximal site for pSc119.2 (120 bp) followed by the signal for pTa794 (5S rDNA), and (*ii*) one site corresponding to pSc119.2 followed by an important block of the repetitive family of 480 bp (pSc74) just following the breakpoint. Neither of these DNA families (120 and 480 bp) are characteristic of the telomeres of chromosome 5R^m. Further, the added region does not show any hybridization to pTa794. All these results point out that the translocated region does not correspond to telomeric segments from 5R^m but to another chromosome. Only the chromosome arms 2R^{m}S, 2R^{m}L, and 7R^{m}L present the tandem formed by the 120–480 bp repetitive family sequences in a distal position. Consequently, these chromosomes are the putative donors of these translocated segments (Figs. 2*m*–2*q*).

Secondary grafted segment

In some plants, another translocated structure affecting the same chromosome was found. In this case, the grafted segment from *S. montanum* was larger than that mentioned above and was interspersed amongst wheat chromatin (Figs. 2*m* and 2*o*), as demonstrated by genomic in situ hybridization. Mapping the DNA sequences in the extra segment showed the following regions (from nearest to the centromere towards the distal end of the chromosome): (*i*) wheat chromatin, including one proximal site for pSc119.2 (120 bp) followed by the signal for pTa794 (5S rDNA), (*ii*) a wide hybridization site for pSc74 (480 bp) flanked by the signal for pSc119.2 (120 bp) that was approximately twice as long as that observed in the distal end of the primary grafted segment, (*iii*) a second tandem formed by pTa794 followed by the signal for pSc119.2, and (*iv*) a short segment of wheat chromatin.

The terminal region of the secondary larger grafted segment seems to be an inverted region of the proximal region. Moreover, the proximal half of the secondary grafted segment looks similar to the primary grafted segment (Figs. 2*m*–2*p*). The most feasible explanation of this is illustrated in Fig. 3. It is assumed that the distal half of the secondary grafted region represents a reversed duplication of the proximal half. This could be the result of a previous process with formation of dicentric chromosomes or chromatids followed by the breakage of the dicentric bridge between the cell poles during anaphase of meiosis and (or) mitosis.

It is of interest to note that plants structurally homozygous for either translocated structure were relatively stable compared with original wheat – *S. montanum* addition lines. The rye–wheat translocation could be important as basic material for alien introgression into wheat.

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References

Bedbrook, J.R., Jones, J., O'Dell, M., Thompson, R.D., and Flavell, R.B. 1980. A molecular description of telomeric heterochromatin in *Secale* species. Cell, **19**: 545–560.

Cuadrado, A., and Jouve, N. 1994. Mapping and organization of highly-repeated DNA sequences by means of simultaneous and sequential FISH and C-banding in 6x-Triticale. Chromosome Res. **2**: 331–338.

Devos, K.M., Atkinson, M.D., Chinoy, C.N., Francis, M.A., Harcourt, R.L., Koebner, R.M.D., Liu, C.J., Masojc, P., Xie, D.X., Gale, M.D. 1993. Chromosomal rearrangements in the rye genome relative to that of wheat. Theor. Appl. Genet. **85**: 673–680.

Dvořák, J., Zhang, H.-B., Kota, R.S., and Lassner, M. 1989. Organization and evolution of the 5S ribosomal RNA gene family in wheat and related species. Genome, **32**: 1003–1016.

Friebe, B., Mukai, Y., Dhaliwal, H.S., Martin, T.J., and Gill, B.S. 1991. Identification of alien chromatin specifying resistance to wheat streak mosaic and greenbug in wheat germplasm by C-banding and in situ hybridization. Theor. Appl. Genet. **81**: 381–389.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Gerlach, W.L., and Dyer, T.A. 1980. Sequence organization of the repeating units in the nucleus of wheat that contain 5S rRNA genes. Nucleic Acids Res. **8**: 4851–4865.

Guidet, F., Rogowsky, P., Taylor, C., Sog, W., and Langridge, P. 1991. Cloning and characterization of a new rye-specific repeated sequence. Genome, **31**: 137–142.

Gustafson, J.P., Evans, L.E., Josifek, K. 1976. Identification of chromosomes in *Secale montanum* and individual *Secale montanum* chromosome addition to 'Kharkov' wheat by heterochromatin bands and chromosome morphology. Can. J. Genet. Cytol. **18**: 339–343.

Heslop-Harrison, J.S., Leitch, A.R., Schwarzacher, T., and Anamthawat-Jonsson, K. 1990. Detection and characterization of 1B/1R translocations in hexaploid wheat. Heredity, **65**: 385–392.

Heslop-Harrison, J.S., Schwarzacher, T., Anamthawat-Jonsson, K., Leitch, A.R., Shi, M., Leitch, I.J. 1991. *In situ* hybridization with automated chromosome denaturation. Technique. J. Methods Cell Mol. Biol. **3**: 109–116.

Jones, J.D.G., and Flavell, R.B. 1982*a*. The mapping of highly-repeated DNA families and their relationship to C-bands in chromosomes of *Secale cereale*. Chromosoma, **86**: 595–612.

Jones, J.D.G., and Flavell, R.B. 1982*b*. The structure, amount and chromosomal localization of defined repeated DNA sequences in species of the genus *Secale*. Chromosoma, **86**: 613–641.

Lapitan, N.L.V., Sears, R.G., Rayburn, A.L., and Gill, B.S. 1986. Wheat–rye translocations. Detection of chromosome breakpoints by in situ hybridization with a biotin-labeled DNA probe. J. Hered. **77**: 415–419.

Lapitan, N.L.V., Sears, R.G., and Gill, B.S. 1987. Amplification of repeated DNA sequences in wheat × rye hybrids regenerated from tissue culture. Theor. Appl. Genet. **75**: 381–388.

Le, H.T., Armstrong, K.C., and Miki, B. 1989. Detection of rye DNA in wheat–rye hybrids and wheat translocation stocks using total genomic DNA as probe. Plant Mol. Biol. Rep. **7**: 150–157.

Leitch, I.J., Leitch, A.R., Heslop-Harrison, J.S. 1991. Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. Genome, **34**: 329–333.

Lukaszewski, A., and Gustafson, J.P. 1983. Translocation and modifications of chromosomes in triticale × wheat hybrids. Theor. Appl. Genet. **64**: 239–248.

McIntyre, C.L., Pereira, S., Moran, L.B., and Appels, R. 1990. New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. Genome, **33**: 635–640.

Montero, M., Sanz, J., and Jouve, N. 1986. Meiotic pairing and alpha-amylase phenotype in a 5B/5R^{m} *Triticum aestivum – Secale montanum* translocation line in common wheat. Theor. Appl. Genet. **73**: 122–128.

Mukai, Y., and Gill, B.S. 1991. Detection of barley chromatin added to wheat by genomic *in situ* hybridization. Genome, **34**: 448–452.

Mukai, Y., Endo, T.R., and Gill, B.S. 1990. Physical mapping of the 5S rRNA multigene family in common wheat. J. Hered. **81**: 290–295.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.

Mukai, Y., Friebe, B., and Gill, B.S. 1992. Comparison of C-banding patterns and in situ hybridization sites using highly repetitive and total genomic rye DNA probes of Imperial rye chromosomes added to Chinese Spring wheat. Jpn. J. Genet. **67**: 71–83.

Mukai, Y., Friebe, B., Hatchett, J.H., Yamamoto, M., and Gill, B.S. 1993*a*. Molecular cytogenetic analysis of radiation-induced wheat–rye terminal and intercalary chromosomal translocations and the detection of rye chromatin specifying resistance to Hessian fly. Chromosoma, **102**: 88–95.

Mukai, Y., Nakahara, Y., and Yamamoto, M. 1993*b*. Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence *in situ* hybridization using total genomic and highly repeated DNA probes. Genome, **36**: 489–494.

Rayburn, A.L., and Gill, B.S. 1985. Use of biotin-labelled probes to map specific DNA sequences on wheat chromosomes. J. Hered. **76**: 78–81.

Rayburn, A.L., and Gill, B.S. 1987. Molecular analysis of the D-genome of the Triticeae. Theor. Appl. Genet. **73**: 385–388.

Schwarzacher, T., Anamthawat-Jonsson, K., Harrison, G.E., Islam, A.K.M.R., Jia, J.Z., King, I.P., Leitch, A.R., Miller, T.E., Reader, S.M., Rogers, W.J., Shi, M., and Heslop-Harrison, J.S. 1992. Genomic *in situ* hybridization to identify alien chromosomes and chromosome segments in wheat. Theor. Appl. Genet. **84**: 778–786.

Shewry, P.R., Parmer, S., and Miller, T.E. 1985. Chromosomal location of the structural genes for the M_r 75.000 ω-secalins in *Secale montanum* Guss.: evidence for a translocation involving chromosomes 2R and 6R in cultivated rye *Secale cereale* L. Heredity, **54**: 381–383.

Stutz, H.C. 1972. On the origin of cultivated rye. Am. J. Bot. **59**: 59–70.

Van Heemert, C., and Sybenga, J. 1972. Identification of the three chromosomes involved in the translocations which structurally differentiate the genome of *Secale cereale* L. from those of *Secale montanum* Guss. and *Secale vavilovii* Grosse. Genetics, **43**: 387–393.

Physical mapping of 5S rDNA loci by direct-cloned biotinylated probes in barley chromosomes

K. FUKUI[1]

Department of Breeding, Hokuriku National Agricultural Experiment Station, Joetsu 943-01, Japan

AND

Y. KAMISUGI AND F. SAKAI

Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba 305, Japan

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5S rDNA loci have been mapped on barley chromosomes by in situ hybridization using five reciprocal translocation lines. Two kinds of DNA probes covering either the 5S rDNA coding region or the 5S rDNA coding and flanking noncoding regions were used. They were prepared by direct cloning from interphase nuclei and simultaneous direct labeling in PCR. Four 5S rDNA loci were detected in a haploid genome by the 5S rDNA coding region, whereas in addition, the four or six 5S rDNA related sites, depending on the variety used, were revealed by the probe covering the flanking region. The four 5S rDNA loci revealed and mapped on the barley chromosomes: 2 (2I), 3 (3I), 1 (7I), and 4 (4I) were designated *5SRrn-I1*, *5SRrn-I2*, *5SRrn-I3* and *5SRrn-I4*, respectively, in descending order of copy number of 5S rRNA genes.

Key words: *Hordeum vulgare*, 5S rDNA, in situ hybridization, direct cloning, direct labeling.

FUKUI, K., KAMISUGI, Y., et SAKAI, F. 1994. Physical mapping of 5S rDNA loci by direct-cloned biotinylated probes in barley chromosomes. Genome, **37** : 105–111.

Des locus d'ADNr de 5S ont été cartographiés sur des chromosomes d'orge par hybridation in situ, utilisant cinq lignées de translocations réciproques. Deux sortes de sondes d'ADN ont été utilisées : celles couvrant la région codant l'ADNr de 5S ou celles couvrant la région codant l'ADNr de 5S et les régions non codantes de flancs. Ces sondes ont été préparées par clonage direct de noyaux en interphase et par le marquage direct simultané dans les réactions de polymérisation en chaîne. Quatre locus d'ADNr de 5S ont été détectés dans un génome haploïde par la région codant l'ADNr de 5S alors qu'en plus, par la sonde couvrant les régions de flancs, quatre ou six sites reliés à l'ADNr de 5S ont été révélés, selon les variétés. Les quatre locus d'ADNr de 5S révélés et cartographiés sur les chromosomes de l'orge : 2 (2I), 3 (3I), 1 (7I), et 4 (4I) ont été respectivement désignés *5SRrn-I1*, *5SRrn-I2*, *5SRrn-I3*, et *5SRrn-I4*, d'après l'ordre décroissant du nombre de copies des gènes d'ADNr de 5S.

Mots clés : *Hordeum vulgare*, ADNr de 5S, hybridation in situ, clonage direct, marquage direct.

[Traduit par la rédaction]

Introduction

The 18S, 5.8S, and 26S ribosomal RNA genes (rDNA) have been studied extensively since the earliest days of molecular cytology, as they exist in large copy number within the genome (Ingle et al. 1975; Appels et al. 1980; Saghai-Maroof et al. 1984). Numerous reports of in situ hybridization (ISH) using rRNA or rDNA probes on barley (Appels et al. 1980), wheat (Appels et al. 1980), and rice (Fukui et al. 1987*a*; Fukui 1990; Iijima et al. 1991) have been made.

For the 5S ribosomal RNA genes (5S rDNA) several reports estimated the number of sites and presented the DNA sequences, including noncoding spacer regions (Gerlach and Dyer 1980; Mascia et al. 1981; Vanderberghe et al. 1984; Scoles et al. 1988; Gottlob-McHugh et al. 1990; Lapitan 1992). There is, however, only limited information on the location of the 5S rDNA loci on the chromosomes. Mukai et al. (1990) physically mapped six 5S rDNA loci on wheat chromosomes in conjunction with deletion mapping. One and two 5S rDNA loci have been detected in tomato (Lapitan et al. 1991) and rye (Reddy and Appels 1989), respectively.

The difficulty of the exact mapping of 5S rDNA loci lies in the fact that the size of a 5S rDNA array within a locus is much smaller than that of an 18S–5.8S–26S rDNA array. Thus more sensitive ISH is required to detect the 5S rDNA locus (e.g., Appels et al. 1980). Moreover, in the case of barley, identifying each chromosome among chromosomes 1 to 5 has proved to be impossible, even by computer imaging technology, without applying banding methods (Fukui et al. 1987*b*). Kolchinsky et al. (1990), thus, used wheat–barley chromosome addition lines in ISH and concluded that only one chromosome possessed a 5S rDNA locus.

We report here the results of physical mapping of 5S rDNA loci in barley chromosomes using biotinylated probes prepared by newly developed methods of sequential application of microdissection (Fukui et al. 1991, 1992; Kamisugi et al. 1993), direct cloning, and direct labeling. Two kinds of probes containing either the 5S rDNA coding region or the coding region with a flanking noncoding region revealed four 5S rDNA loci in four barley chromosomes and four or six 5S rDNA related sites.

Materials and methods

Plant material and chromosome sample preparation

The two barley (*Hordeum vulgare* L. $2n = 14$) varieties of 'New Golden' and 'Chikurin Ibaraki No. 1' (CI-1) were used. Five reciprocal translocation lines, TS8 (4L, 7L), TS17 (2L, 4L), TS36 (2L, 5L), TS42 (1S, 4L), and TS45 (2L, 3L), derived

[1]Corresponding author.

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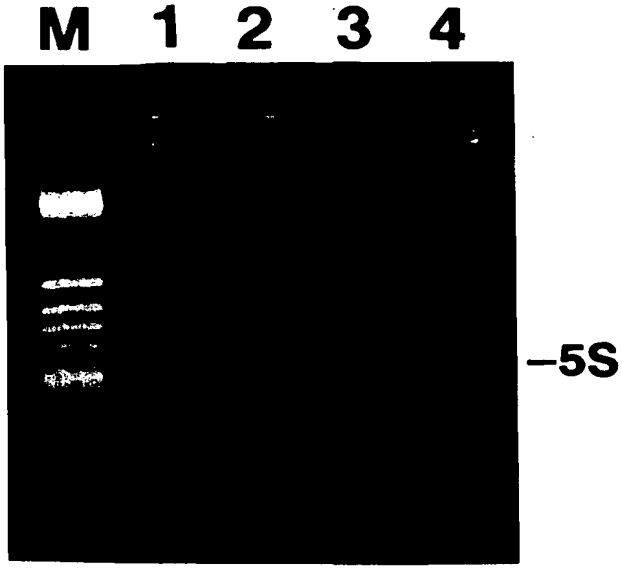

FIG. 1. Amplification of 5S rDNA sequences by the direct cloning method. Lanes 1, 2, and 3 correspond to treatments with approximately 100, 10, and 1 nuclei on a disk in the PCR reaction mixture with the primer set A. Lane 4 is the negative control where a disk without any nucleus was included in the PCR solution. Cycle number was 60 for lanes 1–4. M shows the molecular size markers λ/*Hin*dIII and ϕX174/*Hin*cII.

from CI-1 (Makino 1987, 1988) were used to localize 5S rDNA loci. The numbers indicate the chromosome numbers and the letters S and L indicate the short and long arms, respectively, participating in the reciprocal translocation.

Chromosome samples were prepared by the method described previously (Fukui et al. 1992) with minor modifications. Root tips 1–2 cm in length were excised and fixed in methanol – acetic acid (3:1). They were macerated with an enzymatic mixture and were spread over a tinted polyester membrane laid over the surface on the bottom of a ϕ 35 mm plastic Petri dish. No stain was applied.

Laser dissection, direct cloning, and direct labeling

A standard laser dissection method described by Fukui et al. (1992) was applied to cut out the disks of the polyester membrane on which 1, 10, and approximately 100 barley nuclei were spread. The disks were placed into microcentrifuge tubes (500 μL) using fine forceps. They were pretreated with proteinase (1 mg/mL Proteinase K (Wako Pure Chemicals, Osaka, Japan) with 0.45% Tween 20 and Nonidet P-40 (Sigma) in PCR buffer (Perkin Elmer Cetus)) at 55°C for 1 h.

A standard PCR method using a thermal cycler (Perkin Elmer Cetus) was carried out according to the manufacturer's instructions. Samples were amplified through 30 thermal cycles, each cycle consisting of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. If necessary, PCR products were reamplified for a further 30 cycles as detailed below. For the initial 30 cycles, a 100-μL aliquot reaction mixture containing 2.5 units of Taq polymerase, 0.2 mM DNA substrates with biotin-dUTP, and 1 μM of a primer pair in the PCR buffer was used. For the latter 30 cycles, 10 μL of the initial reaction mixture was used adding 90 μL of the fresh reaction mixture. Two pairs of primers with 20 or 21 bases were used for the direct cloning and simultaneous direct labeling of 5S rDNA. Primer set A (5′-GATCCCATCAGAACTCCGAAG-3′), (5′-CGGTGATTTAGTGCTGGTAT-3′), and primer set B (5′-GGATGCGATCATACCAGCAC-3′), (5′-GGGAATGCAACAC-GAGGACT-3′), corresponded to the 301-bp coding region and flanking spacer regions, and 120-bp coding region, respectively.

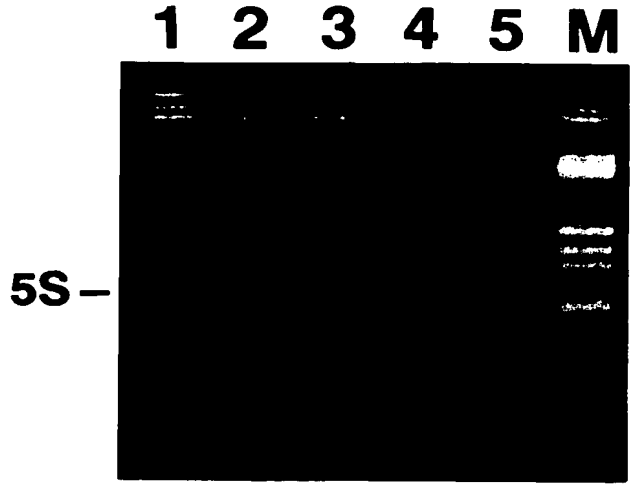

FIG. 2. Simultaneous amplification and labeling of 5S rDNAs. Lanes 1–5 correspond to the substitution percentages of dTTP with biotin-dUTP, 100, 90, 70, 50, 0, respectively. M shows the molecular size markers λ/*Hin*dIII and ϕX174/*Hin*cII. The same primer set was used.

For direct labeling with biotin-11-dUTP, five different combinations of the dTTP with 0, 50, 70, 90, and 100% substitution of biotin-11-dUTP were examined (Weier et al. 1990).

In situ hybridization and image analysis

Slide preparations were treated with 100 μg/mL RNase A (Sigma) in 2× SSC at 37°C for 1 h. The hybridization mixture (4 μg/mL biotin-labeled probe, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 500 μg/mL salmon sperm DNA in 2× SSC) was denatured at 85°C for 10 min and a 30-μL aliquot of the mixture was applied to chromosome samples previously denatured in a 70% formamide solution at 70°C for 1 min. Horseradish peroxidase complex (0.6%, ENZO biochemicals, New York) was applied at 37°C for 30 min. Six hundred microlitres of the mixture of 0.05% DAB (3,3′-diaminobenzidine, Dojindo Laboratories, Kumamoto, Japan) and 0.02% H_2O_2 in PBS was applied at room temperature for 40 min. Finally, the slides were counterstained with a 0.5% Giemsa solution (pH 6.8, Merck). The other details of the experiments were as described by Iijima et al. (1991).

At least three photographs with clear in situ hybridization signals for each variety and translocation line were imported into an image analyzer (VIDAS, Zeiss). The centre positions and the width of the signals were digitally measured. The signal sites were mapped on a quantitative idiogram of barley constructed by imaging methods (Fukui and Kakeda 1990).

Results

Direct cloning, direct labeling of the 5S rDNA probe

Figure 1 shows the results of direct cloning of the coding sequence using the nuclei on the dissected disks as the template. Although the disk with approximately 100 nuclei produced the most obvious band (lane 1), a weaker band was observed from a disk with one nucleus (lane 3). No band appeared in a negative control of a disk without a nucleus (lane 4).

Figure 2 shows the amplified DNAs under five different substitution conditions. Amplification without biotin-dUTP substitution produced a distinct band (lane 5). A fairly high (70% biotin-dUTP) substitution still yielded clear amplified DNA products (lane 3). Therefore, the combination of 30%

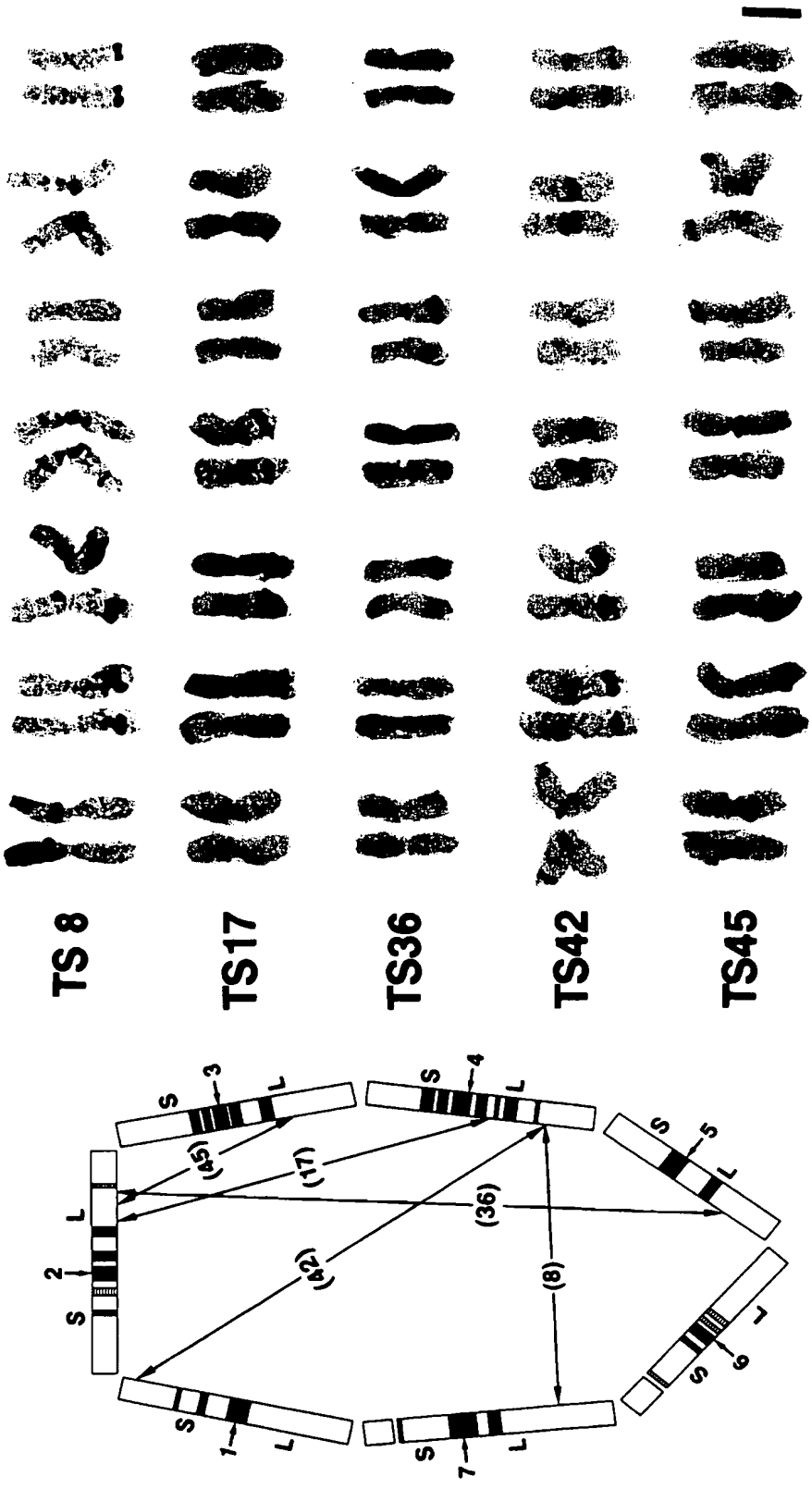

FIG. 3. In situ hybridization of the five translocation lines with the probe covering both the coding and flanking regions. Arrows with numbers beside the chromosomes indicate the centromeric positions and chromosome numbers. Karyograms of respective five translocation lines are shown on the right-hand side. Barley chromosome numbers are displayed underneath. The numbers in the parentheses show the line number. Scale bar = 5 μm.

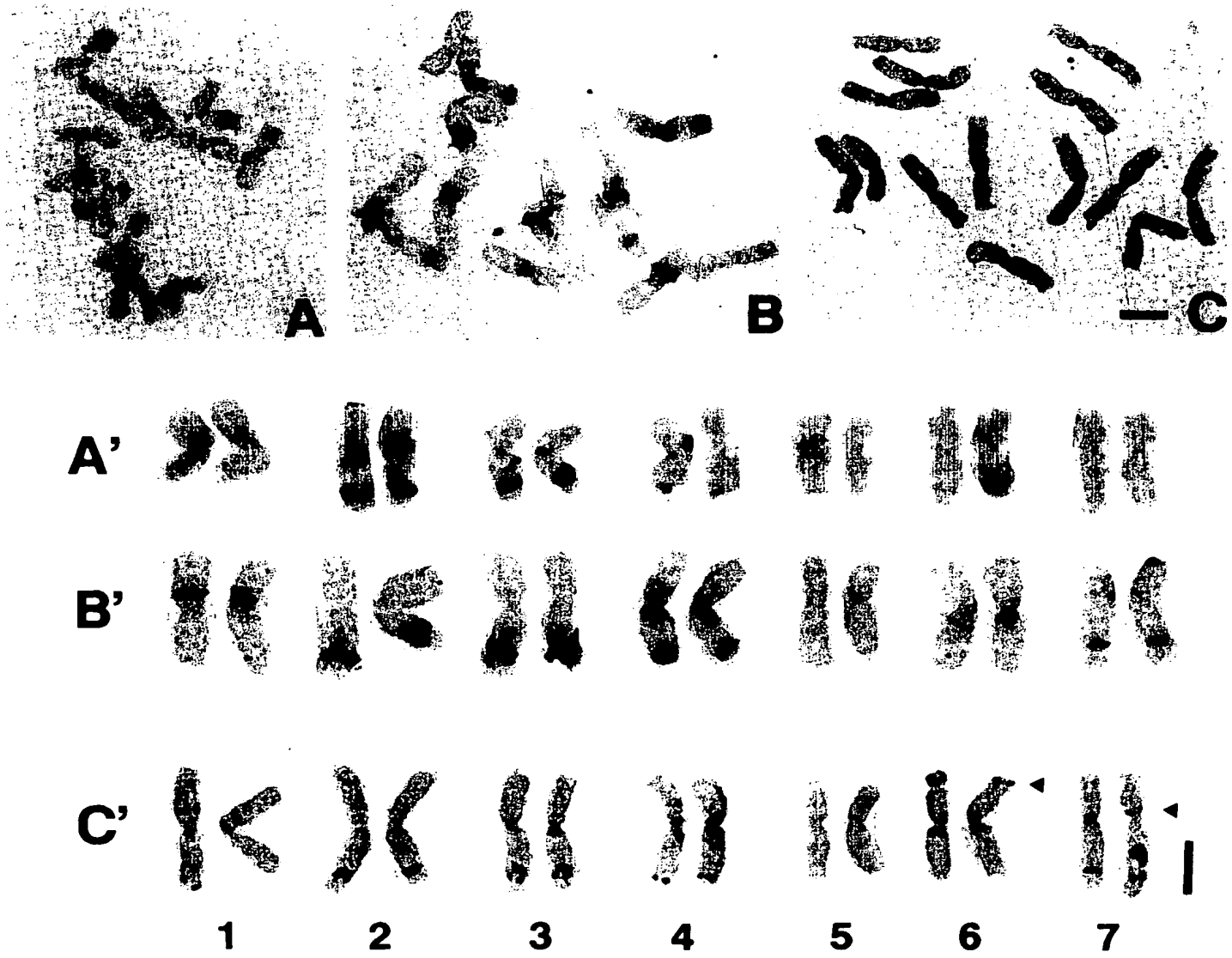

FIG. 4. In situ hybridizations of two barley varieties with two kinds of probes. (A) ISH between CI-1 and the probe of the coding region. (B) ISH between CI-1 and the probe of both the coding and flanking regions. (C) ISH between 'New Golden' and the probe of both the coding and flanking regions. A', B', and C' display the karyograms from the metaphase spreads A, B, and C, respectively. Scale bars = 5 μm.

dTTP and 70% biotin-dUTP was used for probe production throughout the study.

In situ hybridization and mapping 5S rDNA loci

Figure 3 shows chromosomes with signals of the five reciprocal translocation lines by the probe covering both the coding and flanking regions. Reciprocal translocation sites are indicated by lines between the idiograms of the N-banded chromosomes, quantitatively determined by Kakeda (1991). Some of the reciprocal translocations strengthened the morphological features of the chromosomes concerned, such that they were more easily identifiable under the microscope. For example, TS42 has chromosome 4 with the shortest long arm among seven chromosomes within a complement.

Eight pairs of stable signals were observed altogether for all the lines. Chromosomes were completely identified by their morphology and signal patterns. For example, distinct signals appeared in the long arm of chromosomes 2 and 3 of TS8, TS42, and TS45, whereas those distinct signals occurred on chromosomes 3 and 4 for TS17 and on chromosomes 3 and 5 for TS36. Different signal patterns owing to reciprocal translocations occurred between the long arms of chromosomes 2 and 4 of TS17 and between the long arm of chromosomes 2 and 5 of TS36.

Figure 4 shows the results of ISH for the two barley varieties, 'New Golden' and CI-1, with two probes. Figure 4A shows a chromosomal spread of CI-1 with the probe of the coding region. Two pairs of distinct and two pairs of weak signals were observed on the eight chromosomes. Figure 4B shows a chromosomal spread of the same variety hybridized with another probe. The number of the signal sites increased twofold, although all of the additional signals were weak. Eight pairs of signals, two of which were distinct, were counted on the 12 chromosomes. Figure 4C displays ISH between a chromosomal spread of 'New Golden' and the probe covering the coding region and its spacer region. Ten pairs of signals were clearly detected on the 12 chromosomes.

Figures 4A', 4B', and 4C' show karyograms arranged in order of chromosome number. The top and middle arrays show the CI-1 chromosomes with the probe of the coding region and with the probe of coding and spacer regions, respectively. The bottom array shows ISH between 'New Golden' chromosomes and the probe of the coding and spacer regions. Although the strength or the density and size of signals were different among the chromosomes on the middle and bottom arrays, the signal sites and their relative strength within the array or the complement were the same except for two sites: the terminal site of the satellite of chromosome 6 and the proximal site of the short arm of chromosome 7, as indicated by the arrowheads. The former has never occurred in chromosome 6 of CI-1. The latter occasionally occurred at the same site of chromosome 7 of CI-1. It was also clear that the signals on the distal region of chromosome 3L consisted of middle-sized and weak signals (Fig. 4C').

Figure 5 summarizes the signal sites based on the signal pattern that appeared in 'New Golden' chromosomes. Three clear and seven weak signals were recorded altogether. Four signals were displayed by a double circle in chromosomes 2, 3, 1, and 4 (descending order of signal strength), indicating that those were detected by the probe of the coding region. The signal at chromosome 4 was weak, unlike the rest of three signals. Accurate ranking of the signal strength among the rest of the weaker signals detected by the probe with noncoding regions and represented by the solid circles, was difficult.

Chromosome 2 has the strongest signal, occurring at the distal region of its long arm. A complex of the second largest and weak signals is located at the distal region of chromosome 3L. The third largest signal was observed in the proximal region of chromosome 2. A weak and the fourth strongest signal were seen at the proximal and the distal regions of chromosome 4L, respectively. No signal was detected in chromosome 5. A weak signal was identified both at the terminal region of the satellite and the proximal region of chromosome 6L. A weak signal was revealed in the respective proximal and distal regions of chromosome 7S and 7L.

Discussion

Four 5S rDNA loci were accurately mapped on the four barley chromosomes 1, 2, 3, and 4, which correspond to homoeologous groups 7I, 2I, 3I, and 4I, respectively (Jacobsen and von Bothmer 1992). The probe covering both the 120-bp coding region and 181-bp noncoding flanking region hybridized to the 10 sites located on six of the 'New Golden' chromosomes: chromosomes 1, 2, 3, 4, 6, and 7. Four signals were localized on chromosomes 6 and 7 corresponding to homoeologous groups 6I and 5I, respectively.

After the nomenclature of previous rDNA sites, numbers were assigned in the temporal order of discovery (Reddy and Appels 1989; Mukai et al. 1990; Leitch and Heslop-Harrison 1992). The 5S rDNA loci found in chromosomes 2L, 3L, 1S, and 4L, in descending order of copy number, are designated as *5SRrn-I1*, *5SRrn-I2*, *5SRrn-I3*, and *5SRrn-I4*, respectively. The loci that might contain similar DNA sequences to the 5S rDNA flanking noncoding regions, which occurred in chromosomes 3L, 4L, 6S, 6L, 7S, and 7L, were named: *5SRsp-I1*, *5SRsp-I2*, *5SRsp-I3*, *5SRsp-I4*, *5SRsp-I5*, and *5SRsp-I6*, respectively.

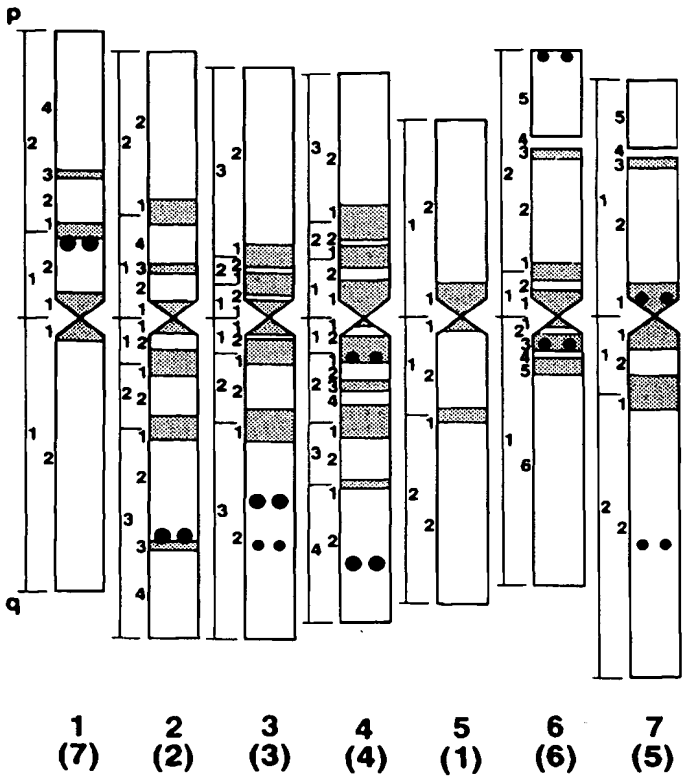

FIG. 5. Mapping of four 5S rDNA loci and the six spacer sequences. The double circles, solid circles, and shaded regions correspond to the 5S rDNA loci, 5S rDNA spacer sequences, and the N-band positive regions, respectively. Allocation of the addresses was after Fukui and Kakeda (1990) with minor modifications. Values in and out of the parentheses under the idiogram indicate the barley chromosomes numbers and homoeologous groups.

In the present study, we report the existence of four 5S rDNA loci in barley. These results contrast with the results of both Appels et al. (1980) and Kolchinsky et al. (1990) who report only one such locus, though the former authors did speculate as to the existence of more minor 5S rDNA loci. Kolchinsky et al. (1990) identified one 5S rDNA locus on barley chromosome 2. In the present work, the strongest signal was observed on chromosome 2L and thus may correspond to the locus reported by Kolchinsky et al. (1990). In this work, however, two medium strength signals of *5SRrn-I2* and *5SRrn-I3* on chromosomes 3L and 1S were detected by highly sensitive ISH methods, as was one weak signal of *5SRrn-I4* on chromosome 4L.

Dvořák et al. (1989) detected two 5S rDNA loci in the diploid species of *Triticum* and *Aegilops*, with the exception of *A. speltoides*, which showed only one 5S rDNA locus. Their results suggested that 5S rDNA may occur on C-band positive regions; however, Mukai et al. (1990) mapped six 5S rDNA loci on six wheat chromosomes and found that 5S rDNA loci did not correspond with any C-band positive regions. In the present study, 5S rDNA loci were confirmed not to occur on the C-band positive regions of barley chromosomes.

Appels et al. (1980) and Mukai et al. (1990) showed that the 5S rDNA loci are linked to 18S–5.8S–26S rDNA loci in wheat and rye. However, the results presented have shown

```
1
GGATGCGATC ATACCAGCAC TAAATCACCG GATCCCATCA GAACTCCGAA

GTTAAGCGTG CTTGGGTGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
                  120 1
AGTCCTCGTG TTGCATTCCC CTTTTTAAAT ATATTTTTGC GCCACGTGAC

AAGGATGACG CGGGAGCGTG ATCTATATGA CCTCATTTTC TTATTTGGGA

CGATTACTGT GTGACTTTTC CCACCGCGCT TGACACCCAA CGACTAGTAG
                                                    181
TAGTAGTAGT AGTAGTGTCA AGCATAAGGA ACAAATAGAT AGTTGCATGTC
```

FIG. 6. The DNA sequences of 5S rDNA coding region (120 bp) and its spacer region (181 bp, underlined). Zero to five bases differed from the sequences of Kolchinsky et al. (1990) and were observed in both coding and spacer regions.

that none of the four 5S rDNA sites occur on the same chromosome in situ hybridizing to the 18S–5.8S–26S coding region probes as Appels et al. (1980) had suggested.

Six signal sites reacted only to the probe that contained the flanking noncoding regions. The DNA homology search of the spacer region (181 bp, Fig. 6, over 75% sequence homology) using the EMBL nucleic acid data bank (DNASIS version 7.0, Hitachi Software Engineering Co., Ltd.) revealed no similar DNA sequences registered in the data base. It may be speculated that the sites are the relics of active loci where most of the 5S rDNA sequences have already been eliminated or their DNA sequences have already changed to a level at which the probe of the coding region could not maintain hybridization under the current hybridization and (or) washing stringency as suggested for barley rDNA loci by Leitch and Heslop-Harrison (1992). In fact, frequent duplications and deletions in the 5S rDNA genes and the associated spacer regions were reported in the family Triticeae (Scoles et al. 1988).

Two new technologies introduced and developed in this study have the following advantages. Firstly, probes for ISH were produced by direct cloning and direct labeling methods from nuclei. This made probe preparation much easier than before. Several interphase or mitotic nuclei prepared by an ordinary cytological method (Fukui and Kakeda 1990; Fukui and Iijima 1991) are sufficient for the template of the PCR. No DNA isolation, purification, or ordinary labeling procedures are necessary.

Secondly, imaging methods (Fukui 1986, 1988) made the quantification of the signal information much more accurate and convenient. By the method reported here, the precise positioning of the centre of the signals on the chromosomes could be ascertained without banding methods. Although the estimation of copy number of the genes from the signal strength still remains crude (Mukai et al. 1988; Fukui 1993), it would be more accurate using imaging methods in the near future.

The advantages of ISH in its ability to quantify signal strength and work reproducibly with single copy sequences will make it an essential tool in biological sciences.

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Appels, R., Gerlach, W.L., Dennis, E.S., Swift, H., and Peacock, W.J. 1980. Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. Chromosoma, **78**: 293–311.

Dvořák, J., Zhang, H.-B., Kota, R.S., and Lassener, M. 1989. Organization and evolution of the 5S ribosomal RNA gene family in wheat and related species. Genome, **32**: 1003–1016.

Fukui, K. 1986. Standardization of karyotyping plant chromosomes by a newly developed chromosome image analyzing system (CHIAS). Theor. Appl. Genet. **72**: 27–32.

Fukui, K. 1988. Analysis and utility of chromosome information by using the chromosome image analyzing system, CHIAS. Bull. Natl. Inst. Agrobiol. Resour. **4**: 153–176.

Fukui, K. 1990. Localization of rRNA genes on rice chromosomes. Rice Biotech. Q. **1**: 18–19.

Fukui, K. 1993. Development of *in situ* hybridization methods. *In* Studies on DNA base sequences in plants. Study report No. 280, Research Council MAFF, Tokyo. pp. 94–98.

Fukui, K., and Iijima, K. 1991. Somatic chromosome map of rice by imaging methods. Theor. Appl. Genet. **81**: 589–596.

Fukui, K., and Kakeda, K. 1990. Quantitative karyotyping of barley chromosomes by image analysis methods. Genome, **33**: 450–458.

Fukui, K., Kakeda, K., Hashimoto, J., and Matsuoka, S. 1987*a*. *In situ* hybridization of ^{125}I labeled rRNA to rice chromosomes. Rice Genet. Newslett. **4**: 114–116.

Fukui, K., Kakeda, K., and Matsuno, T. 1987*b*. Karyotypes of barley chromosomes constructed by several parameters. Barley Genet. **5**: 415–422.

Fukui, K., Minezawa, M., Kamisugi, Y., Yanagisawa, T., Fujishita, M., and Sakai, F. 1991. Microdissection of barley chromosome by the cell work station. Barley Genet. **6**: 272–276.

Fukui, K., Minezawa, M., Kamisugi, Y., Ishikawa, M., Ohmido, N., Yanagisawa, T., Fujishita, M., and Sakai, F. 1992. Microdissection of plant chromosomes by argon ion laser beam. Theor. Appl. Genet. **84**: 787–791.

Gerlach, W.L., and Dyer, T.A. 1980. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Res. **8**: 4851–4865.

Gottlob-McHugh, S.G., Levesque, M., Mackenzie, K., Olson, M., Yarash, D., and Johnson, D.A. 1990. Organization of the 5S rRNA genes in the soybean *Glycine max* (L.) Merrill and conservation of the 5S rDNA repeat structure in higher plants. Genome, **33**: 486–494.

Iijima, K., Kakeda, K., and Fukui, K. 1991. Identification and characterization of somatic rice chromosomes by imaging methods. Theor. Appl. Genet. **81**: 597–605.

Ingle, J., Timmis, J.N., and Sinclair, J. 1975. The relationship between satellite DNA, ribosomal RNA gene redundancy and genome size in plants. Plant Physiol. **55**: 496–501.

Jacobsen, N., and von Bothmer, R. 1992. Supraspecific groups in the genus *Hordeum*. Hereditas, **116**: 21–24.

Kakeda, K. 1991. Studies on new analytic methods for plant chromosomes and their use for genetics and breeding. Ph.D thesis, Kyoto University. pp. 1–108.

Kamisugi, Y., Sakai, F., Minezawa, M., Fujishita, M., and Fukui, K. 1993. Recovery of the dissected C-banded regions of *Crepis* chromosomes. Theor. Appl. Genet. **85**: 825–828.

Kolchinsky, A., Kanazin, V., Yakovleva, E., Gazumyan, A, Kole, C., and Ananiev, E. 1990. 5S-RNA genes of barley are located on the second chromosome. Theor. Appl. Genet. **80**: 333–336.

Lapitan, N.L.V. 1992. Organization and evolution of higher plant nuclear genomes. Genome, **35**: 171–181.

Lapitan, N.L.V., Ganal, M.W., and Tanksley, S.D. 1991. Organization of the 5S ribosomal RNA genes in the genome of tomato. Genome, **34**: 509–514.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by *in situ* hybridization. Genome, **35**: 1013–1018.

Makino, T. 1987. Chromosomal analyses of reciprocal translocation lines in barley. Gamma Field Symp. **26**: 107–121.

Makino, T. 1988. Production of reciprocal translocation lines and genetical analysis of tetravalent behavior in barley. Bull. Inst. Radiat. Breed. **7**: 69–107.

Mascia, P.N., Rubenstein, I., Phillips, R.L., Wang, A.S., and Xiang, L.Z. 1981. Localization of the 5S rRNA genes and evidence for diversity in the 5S rDNA region of maize. Gene, **15**: 7–20.

Mukai, Y., Yamamoto, M., and Fukui, K. 1988. Quantitative estimation of ribosomal RNA genes of wheat chromosomes by *in situ* hybridization using biotin-labeled probes. Jpn. J. Genet. **63**: 582.

Mukai, Y., Endo, T.R., and Gill, B.S. 1990. Physical mapping of the 5S rRNA multigene family in common wheat. J. Hered. **81**: 290–295.

Reddy, P., and Appels, R. 1989. A second locus for the 5S multigene family in *Secale* L.: sequence divergence in two lineages of the family. Genome, **32**: 456–467.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A., and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. U.S.A. **81**: 8014–8018.

Scoles, G.J., Gill, B.S., Xin, Z.-Y., Clarke, B.C., McIntyre, C.L., Chapman, C., and Appels, R. 1988. Frequent duplication and deletion events in the 5S RNA genes and the associated spacer regions in the *Triticeae*. Plant Syst. Evol. **160**: 105–122.

Vanderberghe, A., Chen, M., Dams, E., de Bare, R., de Roeck, E., Huysmans, E., and de Wachter, R. 1984. The corrected nucleotide sequences of 5S RNAs from six angiosperms. FEBS Lett. **171**: 17–23.

Weier, H.G., Segraves, R., Pinkel, D., and Gray, J.W. 1990. Synthesis of Y chromosome-specific labeled DNA probes by in vitro DNA amplification. J. Histochem. Cytochem. **38**: 421–426.

Mol Gen Genet (1992) 231:186–193

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Gene targeting in *Arabidopsis thaliana*

Ursula Halfter, Peter-Christian Morris, and Lothar Willmitzer

Institut für Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, W-1000 Berlin 33, FRG

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Summary. Gene targeting of a chromosomally integrated transgene in *Arabidopsis thaliana* is reported. A chimeric gene consisting of the promoter of the 35S RNA of CaMV, the polyadenylation signal of the octopine synthase gene and the coding region of the bacterial hygromycin phosphotransferase gene (*hpt*), which was rendered non-functional by deletion of 19 bp, was introduced into the genome of *A. thaliana* using *Agrobacterium*-mediated gene transfer. A total of 3.46×10^8 protoplasts isolated from 17 independent transgenic *Arabidopsis* lines harbouring the defective chimeric *hpt* gene were transformed via direct gene transfer using various DNA forms containing only the intact coding region of the *hpt* gene. Out of 150 hygromycin-resistant colonies appearing in the course of these experiments, four were the result of targeted recombination of the incoming DNA with the defective chromosomal locus as revealed by PCR and Southern blot analysis. Comparison with the number of transformants obtained when an *hpt* gene controlled by a promoter and terminator from the nopaline synthase gene was employed results in a maximal ratio of homologous to non-homologous transformation in *A. thaliana* of 1×10^{-4}.

Key words: *Arabidopsis thaliana* – Homologous recombination – Protoplast

Introduction

Transformation techniques have been developed for many different eucaryotic organisms. With the exception of *Saccharomyces cerevisiae* and certain other fungi, DNA introduced into the nucleus appears to integrate essentially randomly without the requirement for long stretches of homology. In mammalian cells this non-homologous or illegitimate recombination occurs 100 to 100000 times more frequently than homologous recombination. However the targeted recombination of incoming DNA offers obvious advantages such as the introduction of precise changes into the structure of endogenous genes thus allowing the analysis of structure-function relationships, or the inactivation of endogenous genes via insertional disruption, permitting study of the biological function of a given gene.

Whereas impressive progress has been achieved with respect to gene targeting of endogenous genes in mammalian systems (Capecchi 1989b; Rossant and Joyner 1989) the development of similar techniques for plant systems is still at a very early stage. Hence, up to now only three reports have appeared describing homologous recombination of foreign DNA with chromosomally located cognate DNA in plants. Using direct DNA transfer into tobacco protoplasts, Paszkowski et al. (1988) were able to repair a defective neomycin phosphotransferase (*npt II*) gene, which was previously introduced into tobacco plants, at a frequency of 1×10^{-4} to 1×10^{-5} (ratio of homologous to non-homologous recombination). The other two reports are of *Agrobacterium*-mediated gene transfer also into tobacco protoplasts: Lee et al. (1990) reported homologous recombination with a mutant acetolactate synthase gene conferring resistance to the sulfonylurea class of herbicides and Offringa et al. (1990) describe the restoration of a defective *npt II* gene. Again the targeting frequency was about 1×10^{-4} to 1×10^{-5}.

Here we describe experiments aimed at establishing techniques allowing gene targeting in a different plant species, *Arabidopsis thaliana*, with its attendant advantages of a small genome, dense restriction fragment length polymorphism (RFLP) map and ease of transformation by *Agrobacterium* or direct gene transfer (for review see Meyerowitz 1989). In order to gain insights into the frequency of homologous recombination in *A. thaliana*, a defective hygromycin phosphotransferase (*hpt*) gene was first introduced into the genome of *A. thaliana* by *Agrobacterium*-mediated gene transfer. The defective *hpt* gene was used subsequently as a target for homologous recombination by direct gene transfer into

Offprint requests to: L. Willmitzer

protoplasts and selection for putative recombinants via their resistance to hygromycin. The experiments described here show that gene targeting in the genome of *A. thaliana* can be achieved with a frequency of 1×10^{-4}.

Materials and methods

Construction of the defective hpt *gene, the targeting DNA and the DNA for control transformation.* The coding region of the bacterial hygromycin phosphotransferase (*hpt*) gene (Gritz and Davies 1983) is borne on a *Bam*HI fragment in the plasmid pGL2 (Karesch et al. 1991) and is controlled by the promoter and the polyadenylation signal of the 35S cauliflower mosaic virus (CaMV) RNA. pGL2-encoded *hpt* was functionally inactivated by a 19 bp deletion created by digestion with *Pst*I and *Nco*I, followed by incubation with T4 DNA polymerase in the presence of all four nucleotides in order to seal sticky ends, and subsequent religation. This modified fragment was isolated by *Bam*HI digestion and inserted into the *Bam*HI site of the expression cassette Bin AR (Höfgen and Willmitzer 1990), thus placing the defective *hpt* gene under the control of the 35S CaMV promoter and the polyadenylation signal of the octopine synthase (*ocs*) gene. The plasmid was transformed into *A. tumefaciens* strain GV 2260 using the freeze-thaw method (Höfgen and Willmitzer 1988) resulting in the strain GV2260::pBinAR-HTPΔ.

To provide the targeting DNA, the *Bam*HI fragment of an intact, wild-type *hpt* gene (i.e. without the *Nco*I-*Pst*I deletion) was cloned into the *Bam*HI site of pUC18 (yielding the plasmid pBhpt) and M13mp18 (yielding the plasmid M13-Bhpt) to enable the isolation of single-stranded DNA. The plasmid pNOS-HPT carrying the intact coding region of the *hpt* gene under the control of both the promoter and the polyadenylation signal of the nopaline synthase (*nos*) gene was used in control transformation experiments.

Construction of transgenic A. thaliana *plants carrying a defective* hpt *gene. Arabidopsis thaliana* genotype C24 was transformed by the *Agrobacterium* strain GV2260::pBinAR-HPTΔ using cotyledon disc transformation as described (Schmidt and Willmitzer 1988). Progeny were selected on glucose-containing MS medium in the presence of 50 μg/ml kanamycin.

Gene targeting experiments. Transformation of protoplasts isolated from different transgenic *A. thaliana* lines was performed by polyethylene glycol (PEG)-mediated direct gene transfer (Damm et al. 1989). The following DNA preparations were used for transformation (amounts are given per 0.3 ml transformation aliquots): (i) 1.6 μg DNA of the isolated (purified by gel electrophoresis) *Bam*HI fragment of the plasmid pBhpt containing the intact coding region of the *hpt* gene; (ii) 7.5 μg of the plasmid pBhpt after digestion with *Bam*HI but without separation of vector and insert; and (iii) 13 μg of single-stranded DNA of M13-Bhpt plasmid. Parallel experiments were performed either in the absence or presence of carrier DNA (15 μg calf thymus DNA).

In order to estimate the total transformation efficiency, control transformation experiments were performed using 7.5 μg undigested DNA from the plasmid pNOS-HPT carrying the intact *hpt* gene under the control of the *nos* promoter. Culture of the protoplasts, selection of calli for resistance against hygromycin and regeneration of intact *Arabidopsis* plants was performed as described (Damm and Willmitzer 1988; Damm et al. 1989).

PCR analysis. Genomic DNA was isolated using the cetyl trimethylammonium bromide (CTAB) method of Murray and Thompson (1980). Genomic DNA or control plasmid (0.5 μg and 15 pg respectively) was subjected to 35 polymerase chain reaction (PCR) cycles using heat-stable *Taq* polymerase (Saiki et al. 1985) in a 50 μl reaction mixture containing 10% dimethyl sulphoxide, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 4.5 mM $MgCl_2$, 0.1% gelatin, 200 μM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and 0.5 μM of each primer. Samples were overlaid with several drops of paraffin to prevent evaporation. In an automatic temperature-shift apparatus (Perkin Elmer, Cetus), samples were initially heated to 94° C for 2 min and in all following cycles to 92° C for 1 min to denature the template, cooled to 60° C for 1 min to anneal the primer and heated to 70° C for 5 min to activate the polymerase. The last cycle was extended by 3 min to ensure completion of all products. The polymerase activity was stopped by cooling down to 25° C. Aliquots of 10 μl of the reaction mix were electrophoresed in 1% agarose gels. Further DNA manipulations and DNA blotting and hybridization techniques were as described (Sambrook et al. 1989; Willmitzer et al. 1982).

Results

General strategy and creation of transgenic A. thaliana *plants containing a defective hygromycin phosphotransferase gene*

We decided to set up a system which would allow the recognition of gene targeting events by the expression of a dominant selectable marker gene in *A. thaliana*. As hygromycin resistance is the most reliable marker in *A. thaliana* (Schmidt and Willmitzer 1988), a chimeric gene consisting of the promoter of the 35S CaMV RNA, the polyadenylation signal of the octopine synthase (*ocs*) gene and the coding region of the bacterial (*hpt*) gene was introduced into *A. thaliana* plants using *Agrobacterium*-mediated transformation. The rationale was to keep this *hpt* gene non-functional and thus supply a chromosomally located target for the homologous recombination experiments. Therefore the coding region of the *hpt* gene was modified by introducing a 19 bp deletion, as shown in Fig. 1, resulting in a frame-shift and a premature stop codon in the translated region. The reason

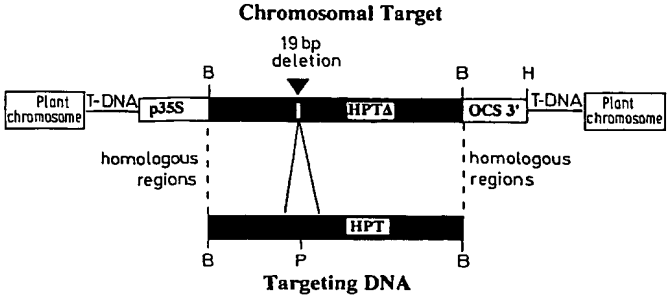

Fig. 1. Structure of the target for homologous recombination, and targeting strategy. The bacterial *hpt* gene controlled by the plant viral 35S promoter and terminated by the *ocs* polyadenylation signal was functionally inactivated by a 19 bp deletion, and the chimeric construct was transferred to *Arabidopsis thaliana*. Different transgenic lines harbouring one to several copies of the defective *hpt* gene were used as recipients for gene targeting experiments. Targeting DNA containing the intact promoterless coding region of *hpt* was used to transform different recipient lines by direct gene transfer. Correction of the defective gene by targeted recombination results in hygromycin-resistant material

for introducing a 19 bp deletion into the coding region was to keep the modification of the target gene rather small, with the intention that this might result in increased frequencies of gene targeting events (Zimmer and Gruss 1989). *Arabidopsis* plants containing this construct were fully sensitive to hygromycin under all conditions tested.

For gene targeting experiments, various DNA sources containing the intact coding region of the *hpt* gene with neither a promoter nor a polyadenylation signal were used. As the result of a successful targeted recombination, it was expected that the incoming DNA containing the intact coding region would recombine with the chromosomally located defective *hpt* gene and thus result in the restoration of a fully functional *hpt* gene where the coding region of the hygromycin gene would be under the control of the 35S promoter and the *ocs* terminator. In this respect it is important to stress that this specific gene (i.e. consisting of the 35S promoter, an intact *hpt* coding region and the *ocs* terminator) had never previously been assembled in vitro in our laboratory. The possibility is thus excluded that the presence of such a gene in transgenic *Arabidopsis* lines could be due to trace contaminations, which is a potentially serious problem especially considering the rather low frequencies of gene targeting observed in plants.

Gene targeting experiments led to a total of 150 hygromycin-resistant calli out of 3.46×10^8 protoplasts

A series of gene targeting experiments was performed aiming at the repair of the chromosomally integrated, defective *hpt* gene with DNA introduced into protoplasts using PEG-mediated direct gene transfer. Hygromycin-resistant calli were selected for and fertile plants regenerated.

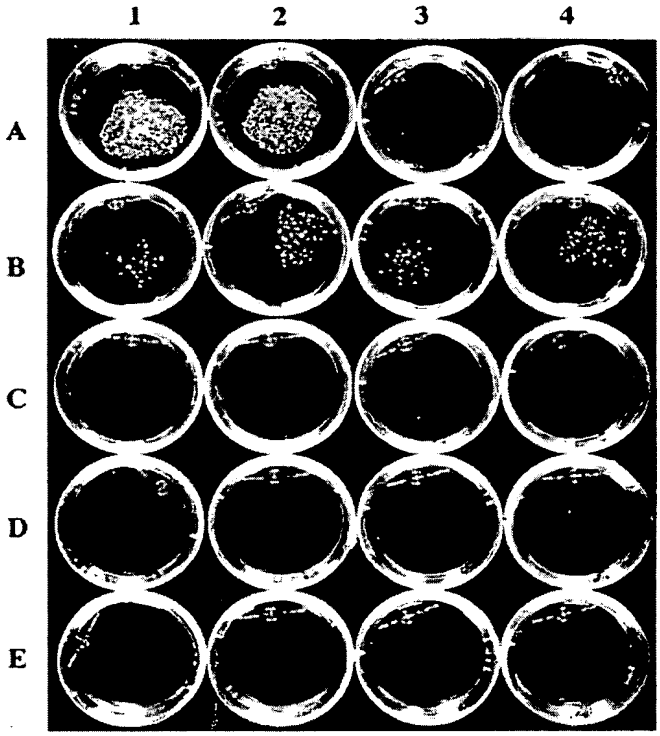

Fig. 2. Appearance of protoplast-derived colonies in different transformation experiments. Row A shows the behaviour of calli derived from non-transformed protoplasts from the target lines under non-selective (1, 2) and selective (3, 4) conditions. Row B shows the outcome of a typical transformation experiment under selective conditions when an intact *hpt* gene (pNOS-HPT) under the control of both the promoter and the polyadenylation signal of the nopaline synthase (*nos*) gene was used. Routinely a relative transformation frequency (macrocalli developing in the presence of hygromycin/macrocalli developing in the absence of hygromycin) of 5% was obtained. Rows C, D and E show typical results of a transformation experiment aimed at gene targeting of the defective chromosomally located *hpt* gene. The very few hygromycin-resistant colonies that appeared were selected for further growth, regeneration and molecular analysis

The following parameters were varied in these experiments:

(a) the transgenic *Arabidopsis* line serving as recipient in the targeting experiments; 17 independently transformed lines harbouring between one and seven copies of the defective *hpt* gene were used.

(b) the nature of the targeting DNA; the *Bam*HI fragment containing the intact coding region of the *hpt* gene isolated from the plasmid pBhpt was used in order to provide only homologous DNA with no non-homologous sequences attached. The entire *Bam*HI-digested plasmid pBhpt was also used both in the presence and the absence of carrier DNA which has been shown to increase the transformation frequency (Damm et al. 1989). As single-stranded (ss) DNA has also been demonstrated to lead to efficient transformation in plant systems (Rodenburg et al. 1989; Halfter 1990), ss DNA isolated from the phage M13-Bhpt, was also used with and without carrier DNA in targeting experiments.

Table 1. Summary of all targeting experiments performed

HPTΔ line	Total no. ($\times 10^6$)	Number of calli regenerated under hygromycin selection			RRF	Recombinant
		PCR_{tested}	$PCR_{pos.}$	Nos-HPT^r		
P 1	22	27		3500		
P 6	11	9		1700		
P 8	12			1900		
P 12	6			1000		
P 15	34	24		5800		
P 19	7			1100		
P 22	25	3		4000		
P 23	39	17	2	6200	3×10^{-4}	R 11, R 15
P 24	2			300		
P 25	28	5		4500		
P 27	52	20	1	8600	1×10^{-4}	R 10
P 31	43	6		6900		
P 32	23	26		3700		
P 37	18	12	1	2900	3.4×10^{-4}	R 46
P 40	10	1		1600		
P 48	6			1300		
P 49	8			1300		
Total	346	150	4	56300	7.1×10^{-5}	

Seventeen different transgenic *Arabidopsis thaliana* lines (HPTΔ line, P 1–P 49) harbouring the defective *hpt* gene were used as protoplast sources for gene targeting experiments
Total no., total number of protoplasts from each target line ($\times 10^6$)
PCR_{tested}, number of hygromycin-resistant calli used as DNA source for the PCR reaction using primers U1 and U2 (see Fig. 3); PCR_{pos}, number of calli resulting in a positive PCR reaction; NOS-HPT^r, number of calli regenerated under selective conditions upon transforming the same number of protoplast used in targeting experiments with the plasmid pNOS-HPT
RRF, relative recombination frequency expressed as a ratio of gene targeting events compared to illegitimate recombination events
Recombinant, name of the identified recombinant lines
Total, the amount of protoplasts and calli are summed over all the experiments performed

Figure 2 shows the effectiveness of the hygromycin selection when applied to mock-transformed regenerated calli from *A. thaliana* protoplasts of the target line. No calli survive the hygromycin selection despite the fact that microscopic analysis indicated a very high frequency of initial cell division (up to 30% of the protoplasts embedded started to develop microcalli before selection with hygromycin was applied). This is in contrast to the appearance of numerous hygromycin-resistant calli in transformation experiments in which pNOS-HPT DNA carrying an intact *hpt* gene was used. A typical result of a gene targeting experiment is shown in the third to fifth rows of Fig. 2. Very few, if any, calli develop in the presence of hygromycin; thus from a large number of experiments involving a total of 3.46×10^8 protoplasts, 150 hygromycin-resistant calli were obtained. A summary of the results of the gene targeting experiments is shown in Table 1.

Molecular analysis of the hygromycin-resistant calli

The appearance of hygromycin-resistant clones can be explained in several ways:
(a) False positives which escaped hygromycin selection. Based on previous experiments this number is expected to be very low (Damm et al. 1989).
(b) Integration into non-homologous sequences resulting in transcriptional and/or translational fusions between the *hpt* coding region and endogenous genes expressed at the callus level. This event, most probably due to preferential insertion of exogenous DNA into transcriptionally active DNA, has been frequently observed in tobacco and *A. thaliana* transformation experiments using both *Agrobacterium*-mediated and direct gene transfer of promoterless constructs (Koncz et al. 1989; Mayerhofer et al. 1991).
(c) Homologous recombination with the defective chromosomal *hpt* gene resulting in restoration of an intact *hpt* gene.

In order to differentiate between these possibilities, DNA was extracted from all 150 hygromycin-resistant calli and analysed by polymerase chain reaction (PCR) (Kim and Smithies 1988). For this purpose two primers were used, one of them covering positions −109 to −123 of the 35S CaMV promoter and offering specificity for the target DNA and the second one including the sequence of 19 nucleotides, which was deleted in the chromosomally defective derivative, and so supplying specificity for the targeting DNA. Both primers would be available for PCR only in cases where hygromycin-resistant clones had resulted from gene targeting events that restore the 19 bp deletion in the defective

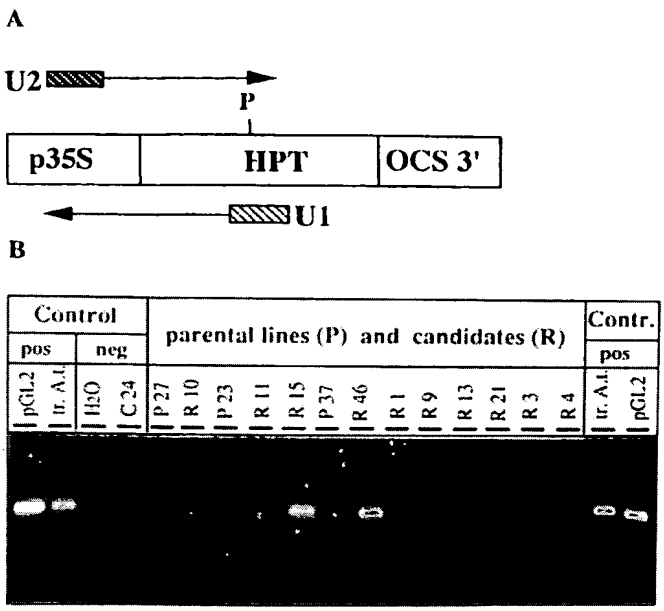

Fig. 3A and B. Gene targeting identified by screening for the linkage of the 35S CaMV promoter to an intact *hpt* gene. **A** Hygromycin-resistant colonies that appeared after gene targeting experiments were screened for restoration of the *hpt* coding sequence and linkage of the restored *hpt* gene to the 35S CaMV promoter by PCR analysis. For this purpose, two oligonucleotide primers were used (*shaded boxes*); one primer (U1) corresponds to the 19 bp sequence deleted in the defective *hpt* target gene (containing the *PstI* site, P) and the other primer (U2) is located within the 35S promoter. Only in the case of a successful gene targeting experiment would both sequences be linked, thus resulting in amplification of a 500 bp long fragment. (Primer sequences: U1, 5'-GGCCTCCGCGACCGGCTGCAGAACAGC-3', U2, 5'-GACGTTCCAACCACGTCTTCAAAGC-3'). **B** Gel electrophoretic separation of PCR products obtained from the following template DNAs: the two outermost left and right lanes contain the results of reactions using pGL2 (plasmid DNA containing 35S-*hpt* sequences) or genomic *A. thaliana* DNA transgenic for pGL2 (tr.A.t.) as template (positive control). The fidelity of the PCR reactions was tested by using wild-type (C24) genomic DNA and by omitting any template DNA (H_2O) (negative controls). The middle part of the gel contains the products obtained by PCR of template DNA from different target lines (P) and candidates for recombination (R)

hpt gene; this should result in the amplification of a 500 bp DNA fragment, as illustrated in Fig. 3.

The fidelity of the PCR analysis was checked uing genomic DNA from non-transformed *A. thaliana* plants, and from the target lines containing the defective *hpt* gene as negative controls. DNA extracted from a line transgenic for the plasmid pGL2, which harbours 35S-*hpt* sequences, served as a positive control and resulted in amplification of a 500 bp fragment (Fig. 3). Upon testing DNA extracted from the 150 calli displaying hygromycin resistance, the expected band of 500 bp in length was seen for four of the transformants. In three of these four instances the targeting DNA was applied in the form of digested plasmid in the presence of carrier DNA, and in one example ss DNA was used. In all other 146 cases tested no band of the expected size was seen, as shown in Fig. 3 and Table 1, indicating that the hygromycin resistance observed for these calli is most likely to be due to illegitimate integration of the *hpt* gene and expression of transcriptional and/or translational fusions.

Further confirmation of the targeting event was obtained by Southern blot analysis. Total DNA isolated from the hygromycin-resistant PCR-positive clones (R) as well as DNA isolated from the corresponding recipient "parental" lines (P) was digested with *Hin*dIII and subjected to gel electrophoresis. (Clone R11 was lost due to fungal contamination in tissue culture). *Hin*dIII digestion of the plant DNA should result in the release of a border fragment spanning the junction between the left part of the T-DNA and the neighbouring plant DNA and thus be unique for each integrated T-DNA copy. This fragment should hybridize to the 35S promoter, the *hpt* coding region and the *ocs* terminator as shown in Fig. 4.

By hybridizing with the *ocs* probe, an additional band is clearly visible in the target line P37 and the recombinant R46, which also appears as a faint band when using the *hpt* probe after longer exposure of the autoradiograph. However the 35S probe, even after prolonged exposure, did not result in a signal. This result can be explained by a partial transfer of the target T-DNA to the plant genome. New bands are also detectable in blots of lines R10 and R15 probed with the *hpt* fragment, which are absent in the recipient lines. This demonstrates that further copies of the (promoterless) *hpt* gene integrated via illegitimate recombination in addition to the targeted recombination. In the case of line R46 no evidence for additional *hpt* sequences was obtained. However in the event of targeted recombination, no additional fragments that hybridize to all of the three probes (i.e. 35S promoter, *ocs* terminator and *hpt* fragment) should be seen in the recipient line. This was true for all three lines (Fig. 4), thus confirming the result obtained by PCR analysis.

The targeted recombination of the *hpt* coding region should also result in restoration of the *PstI* site contained in the 19 bp deletion. DNA from the target line amplified by PCR using specific primers located in the 35S promoter and downstream of the 19 bp deletion could not be digested with *PstI*; however, in the case of the corrected lines, amplified DNA could be digested as shown in Fig. 5, further proving the restoration of the 19 bp deletion. Apparent incomplete digestion of the PCR amplified fragment was observed; a double band differing by the 19 bp and corresponding to the defective and corrected targets was amplified, due to the homozygous genotype in the target line P23.

Taken together, the results obtained by PCR, Southern analysis and restriction analysis show that the corrected *hpt* gene is linked to a 35S promoter and *ocs* terminator, thus proving the construction of this new chimeric gene in planta.

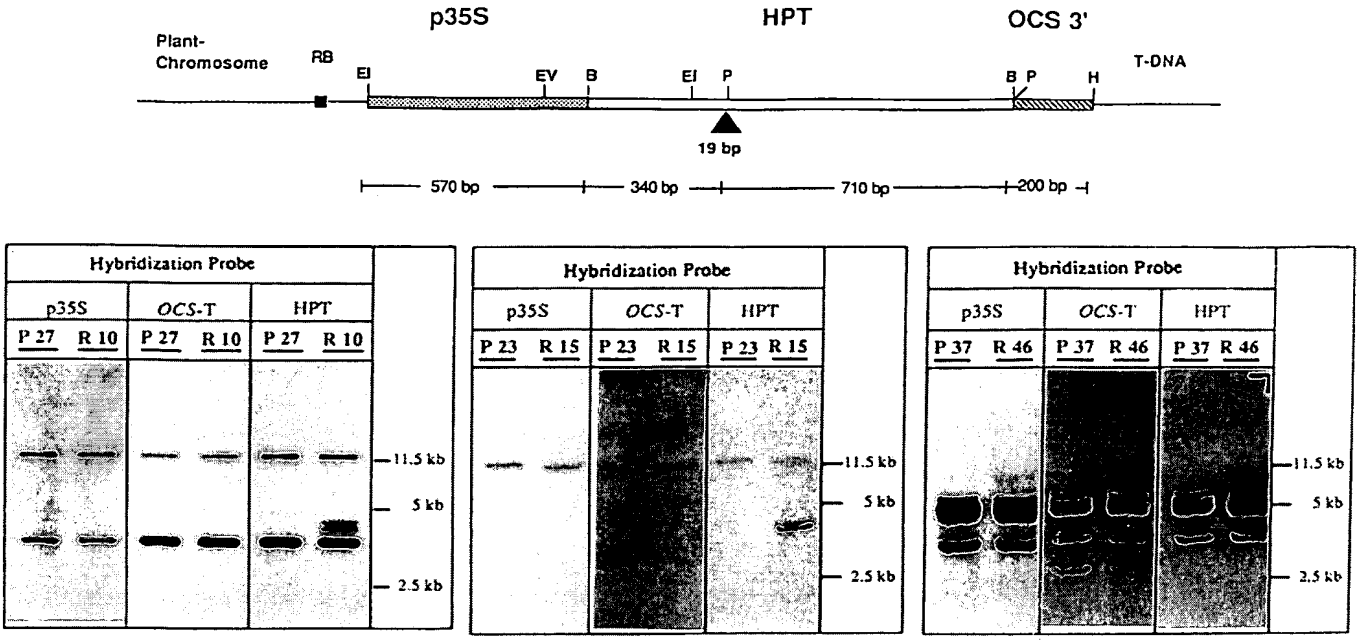

Fig. 4. Southern blot analysis of PCR-positive lines and the corresponding target lines. Total isolated DNA was digested with *Hin*dIII and probed with a 35S promoter fragment (p35S), the terminator of the octopine synthase gene (*OCS*-T) and the coding region of the *hpt* gene (HPT). In the case of a gene targeting event, both target and genuine homologous recombinants should exhibit the same hybridization pattern with the promoter and terminator probe. See the text for further explanation

Discussion

Evidence is presented here that gene targeting in *A. thaliana* can be achieved. Using a highly efficient system for direct DNA transfer into protoplasts (Damm et al. 1989), we have been able to repair a 19 bp deletion in the coding region of a chromosomally located *hpt* gene fused to the 35S CaMV promoter and the *ocs* polyadenylation signal. As a result of homologous recombination between the chromosomally located target and the incoming DNA, the deletion was expected to be replaced by the corresponding sequence of the intact gene. On the one hand, this should result in the restoration of an intact *hpt* gene, giving rise to hygromycin-resistant calli which could be selected. On the other hand this should also result in linking the 19 bp sequence missing in the defective *hpt* gene to the 35S CaMV promoter, thus allowing identification by the use of suitable primers (i.e. one covering part of the 35S CaMV promoter, another including the 19 bp deletion) of true recombinants via PCR screening.

The data shown here demonstrate that four out of 150 hygromycin-resistant calli obtained in the course of these experiments displayed the expected PCR fragment, thus providing evidence of repair of the defective chromosomal *hpt* gene via the incoming DNA. This interpretation is further supported by the results of the Southern blot analysis. The defective gene consisting of the 35S promoter, the *hpt* coding region and the *ocs* terminator does not contain an endogenous *Hin*dIII site. Upon digestion with *Hin*dIII, each gene will thus be liberated as one band together with the left hand T-DNA border, thus creating size-specific fragments for every insertion locus. In the three restored lines analysed, no additional *Hin*dIII fragments that hybridize to all three probes for the 35S promoter, the *hpt* gene and the *ocs* terminator were detectable when compared with the corresponding target lines. The only possibility for the creation of the linkage between the 35S promoter sequences and the sequences deleted in the target gene is by targeted recombination of the intact coding region of the *hpt* gene with the chromosomally located defective *hpt* gene. The intact 35S-*hpt*-*ocs* chimeric gene has never been constructed in our laboratory and could only be constructed in planta by homologous recombination; thus, the possibility of a trace contamination is excluded, as discussed in the other cases where homologous recombination in plants has been reported (Lee et al. 1990). In addition, it has been demonstrated by restriction analysis of PCR-amplified DNA that the *Pst*I site missing in the deleted target DNA has been restored in the corrected lines.

All data obtained are thus in agreement with the interpretation that in these three cases the defective *hpt* gene has been corrected by the incoming DNA. The data obtained do not allow us to differentiate between gene conversion or homologous recombination as the underlying mechanism involved. A total of 3.46×10^8 protoplasts derived from 17 independently transformed *A. thaliana* transformants harbouring the defective *hpt* gene have been used in the process of the experiments described here. Four lines were identified where the defective *hpt* gene was corrected via the targeting DNA. This amounts to a total frequency of 7×10^{-5} if averaged over all experiments.

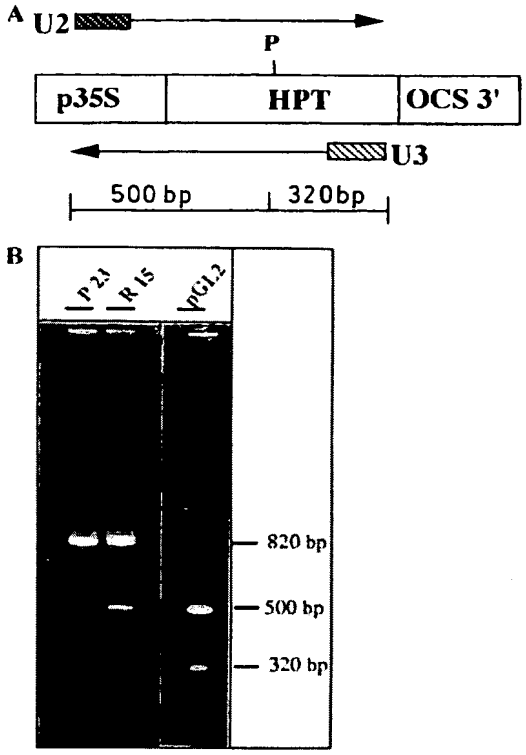

Fig. 5A and B. Correction of the non-functional *hpt* gene by targeting results in the restoration of the internal *PstI* site. **A** The digestion was performed after amplification of an appropriate fragment by PCR to overcome problems due to methylation sensitivity of *PstI* when using genomic DNA. PCR oligonucleotide primers U2 and U3 and their transcriptional orientations are indicated as *shaded boxes* and *arrows*. (Primer sequences: U2, see Fig. 3, U3, 5'-CGCTCCAGTCAATGACCGCTGTTAT-3'. **B** Digested fragments were subjected to electrophoresis. Target line (P23) and the corresponding recombinant (R15) compared with respect to digestibility with *PstI* of the synthesized PCR fragment. pGL2 serves as a positive control

Due to the low number of recombinants, no significant conclusion can be drawn with respect to the importance on recombination frequencies of the various parameters checked (e.g. nature of the targeting DNA, different transgenic lines). Nevertheless it is intriguing that only three out of 17 lines gave rise to gene targeting. These three lines did not differ from other lines with respect to, for example, copy number. Thus as deduced from Fig. 4, hybridization with the 35S promoter fragment gave 1, 2 and 7 bands (indicating the same number of copies of the T-DNA) in the three lines in which recombinants were identified, which is not different from the distribution in the remaining 14 lines that had not given rise to recombinants. If only these three lines are taken into consideration, a much higher frequency of between 1 and 3×10^{-4} is obtained for the homologous recombination event. A similar observation has been reported by Paszkowski et al. (1988) who compared two transgenic tobacco lines harbouring a defective *nptII* gene. In only one line was successful gene targeting obtained. The influence of different target gene loci may be due to different chromatin structures, as discussed by Zijlstra et al. (1989).

With regard to plant systems, three instances of gene targeting in tobacco have been reported: either direct gene transfer targeted at a defective transgene (Paszkowski et al. 1988), or *A. tumefaciens*-mediated transformation targeted at an endogenous gene (Lee et al. 1990) or a transgene (Offringa et al. 1990). The ratio of homologous versus illegitimate recombination observed is in the same range as that described here, i.e. between 4×10^{-4} and 5×10^{-5}. These data indicate that neither the technique used to transfer the targeting DNA (i.e. *A. tumefaciens*-mediated or direct gene transfer) nor whether the target gene is a transgene or an endogenous gene seem to be of dramatic influence with respect to the frequency of homologous recombination. Furthermore a result not necessarily expected is that about the same frequencies are obtained in both tobacco and *A. thaliana* irrespective of the fact that these species differ in genome size by a factor of about 20. Thus genome size does not seem to be of major importance with respect to targeting frequencies as has been also demonstrated by Zheng and Wilson (1990) in animal cells.

In mammalian systems, frequencies for homologous versus illegitimate recombination vary between 10^{-2} and 10^{-7}. The main parameters influencing the frequency are the length of the homologous DNA, the nature of the target gene and the method of introducing the DNA. Thus Capecchi (1989a) showed a correlation between the frequency of gene targeting and the length of homology between the chromosomal target gene and the targeting DNA. Microinjection into the nucleus and electroporation seem to be superior to PEG-mediated transformation techniques. Obviously these parameters still need thorough investigation in plant systems; however, one should bear in mind that microinjection into the nucleus currently represents a major technical challenge with respect to plant systems in general and probably even more so for *A. thaliana* (Crossway et al. 1986; Reich et al. 1986). In conclusion, data are presented that clearly demonstrate the feasibility of achieving gene targeting in *A. thaliana* using direct gene transfer into protoplasts. In order to make this method applicable on a routine basis, the frequencies of homologous versus non-homologous recombination have to be increased. Furthermore strategies have to be devised which also allow the early recognition of homologous recombination occurring with endogenous plant genes.

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References

Capecchi MR (1989a) Altering the genome by homologous recombination. Science 244:1288–1292

Capecchi MR (1989b) The new mouse genetics: altering the genome by gene targeting. Trends Genet 5:70–76

Crossway A, Oakes JV, Irvine JM, Ward B, Knauf VC, Shewmaker CK (1986) Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Mol Gen Genet 202:179–185

Damm B, Willmitzer L (1988) Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. Mol Gen Genet 213:15–20

Damm B, Schmidt R, Willmitzer L (1989) Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. Mol Gen Genet 217:6–12

Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of the hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188

Halfter U (1990) PhD Thesis, Free University of Berlin

Höfgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acid Res 16:9877

Höfgen R, Willmitzer L (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). Plant Sci 66:221–230

Karesch H, Bilang R, Mittelsten-Scheid O, Potrykus I (1991) Direct gene transfer to protoplasts of *Arabidopsis thaliana*. Plant Cell Rep 9:571–574

Kim HS, Smithies O (1988) Recombinant fragment assay for gene targeting based on the polymerase chain reaction. Nucleic Acid Res 16:8887–8903

Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Körber H, Redei GP, Schell J (1989) High frequency T-DNA mediated gene tagging in plants. Proc Natl Acad Sci USA 86:8467–8471

Lee KY, Lund P, Lowe K, Dunsmuir P (1990) Homologous recombination in plant cells after *Agrobacterium*-mediated transformation. The Plant Cell 2:415–425

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Hohn B, Koncz C (1991) T-DNA integration: a mode of illegitimate recombination in plants. EMBO J 10:697–704

Meyerowitz EM (1989) *Arabidopsis*, a useful weed. Cell 56:263–269

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res 8:4321–4325

Offringa R, de Groot MJA, Haagsman HJ, Does MP, van den Elzen PJM, Hooykaas PJJ (1990) Extrachromosomal recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. EMBO J 9:3077–3084

Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7:4021–4026

Reich TJ, Iyer VN, Miki BL (1986) Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti plasmid. Bio/Technology 4:1001–1004

Rodenburg KW, Groot MJA, Schilperoort RA, Hooykaas PJJ (1989) Single stranded DNA used as an efficient new vehicle for transformation of plant protoplasts. Plant Mol Biol 13:711–719

Rossant J, Joyner A (1989) Towards a molecular-genetic analysis of mammalian development. Trends Genet 5:277–283

Saiki RK, Scharf SJ, Faaloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Primer-directed enzymatic amplification of DNA with a thermostable DNA poymerase. Science 230:1350–1354

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schmidt R, Willmitzer L (1988) High efficiency *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. Plant Cell Rep 7:583–586

Willmitzer L, Simons G, Schell J (1982) The Ti-DNA in octopine crown gall tumors codes for seven well-defined polyadenylation transcripts. EMBO J 1:139–146

Zheng A, Wilson J (1990) Gene targeting in normal and amplified cell lines. Nature 344:170–173

Zijlstra M, Li E, Sajjadi F, Subramani S, Jaenisch R (1989) Germ line transmission of a disrupted β2-microglobulin gene produced by homologous recombination in embryogenic stem cells. Nature 342:435–438

Zimmer A, Gruss P (1989) Production of chimaeric mice containing embryonic stem (ES) cells carrying a homeobox *Hox 1.1* allele mutated by homologous recombination. Nature 338:150–153

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CHROMOSOMA

Distribution of 5S and 18S-28S rDNA loci in a tetraploid cotton (*Gossypium hirsutum* L.) and its putative diploid ancestors

Robert E. Hanson[1], M. Nurul Islam-Faridi[1], Edward A. Percival[2], Charles F. Crane[1], Yuanfu Ji[1], Thomas D. McKnight[3], David M. Stelly[1], H. James Price[1]

[1] Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474, USA
(e-mail: monosom@rigel.tamu.edu)
[2] USDA Agricultural Research Service, College Station, TX 77845, USA
[3] Department of Biology, Texas A&M University, College Station, TX 77843, USA

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Abstract. The most widely cultivated species of cotton, *Gossypium hirsutum,* is a disomic tetraploid ($2n=4x=52$). It has been proposed previously that extant A- and D-genome species are most closely related to the diploid progenitors of the tetraploid. We used fluorescent in situ hybridization (FISH) to determine the distribution of 5S and 18S-28S rDNA loci in the A-genome species *G. herbaceum* and *G. arboreum*, the D-genome species *G. raimondii* and *G. thurberi,* and the AD tetraploid *G. hirsutum.* High signal-to-noise, single-label FISH was used to enumerate rDNA loci, and simultaneous, dual-label FISH was used to determine the syntenic relationships of 5S rDNA loci relative to 18S-28S rDNA loci. These techniques provided greater sensitivity than our previous methods and permitted detection of six new G. *hirsutum* 18S-28S rDNA loci, bringing the total number of observed loci to 11. Differences in the intensity of the hybridization signal at these loci allowed us to designate them as major, intermediate, or minor 18S-28S loci. Using genomic painting with labeled A-genome DNA, five 18S-28S loci were localized to the *G. hirsutum* A-subgenome and six to the D-subgenome. Four of the 11 18S-28S rDNA loci in *G. hirsutum* could not be accounted for in its presumed diploid progenitors, as both A-genome species had three loci and both D-genome species had four. *G. hirsutum* has two 5S rDNA loci, both of which are syntenic to major 18S-28S rDNA loci. All four of the diploid genomes we examined contained a single 5S locus. In *G. herbaceum* (A_1) and *G. thurberi* (D_1), the 5S locus is syntenic to a major 18S-28S locus, but in *G. arboreum* (A_2) and *G. raimondii* (D_5), the proposed D-genome progenitor of *G. hirsutum*, the 5S loci are syntenic to minor and intermediate 18S-28S loci, respectively. The multiplicity, variation in size and site number, and lack of additivity between the tetraploid species and its putative diploid ancestors indicate that the behavior of rDNA loci in cotton is nondogmatic, and considerably more complex and dynamic than previously envisioned. The relative variability of 18S-28S rDNA loci versus 5S rDNA loci suggests that the behavior of tandem repeats can differ widely.

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Correspondence to: R.E. Hanson

Introduction

There are approximately 50 species in the cotton genus *Gossypium*, including 45 diploids and at least 5 tetraploids (Fryxell 1992). Collectively, they grow throughout tropical and subtropical parts of the world and are genomically diversified in relation to their geographic ranges (Skovsted 1934). Seven major diploid genomic groups of *Gossypium* have been identified and are designated by the letters A through G (Beasley 1940, 1942; Edwards and Mirza 1979).

The species *Gossypium hirsutum* is an AD disomic tetraploid ($2n=4x=52$) that originated from an interspecific hybridization event(s) between diploid *Gossypium* species thought to be closely related to the A_1-genome species *G. herbaceum* and the D_5-genome species *G. raimondii* (Endrizzi et al. 1985).The *G. hirsutum* chromosomes of A-genome origin are larger, as a group, and are numbered 1–13, whereas those of D-genome origin are numbered 14–26. The A-genome and D-genome diploids are estimated to have diverged from a common ancestor between 6 and 11 million years ago, and the hybridization event(s) leading to *G. hirsutum* is estimated to have occurred 1 to 2 million years ago (Wendel 1989; Wendel and Albert 1992). Previous efforts at interspecific comparisons within *Gossypium* have employed morphological (Fryxell 1992), meiotic (Menzel 1954), karyotypic (Beasley 1940), genetic and molecular criteria (Wendel 1989).

In this study, we have used fluorescent in situ hybridization (FISH) to examine the distribution of 5S and 18S-28S rDNA sites in *G. hirsutum* and closely related diploid species in order to address three objectives. The first objective was to determine the respective homoeologous relationship of chromosomes that bear 5S and

18S-28S rDNA sequences in the diploids. The second objective was to provide new molecular cytogenetic landmarks that will facilitate ongoing construction of a FISH-based karyotype for cotton, and development of an integrated map of recombinational and physical distances among loci defined by molecular markers and cytogenetic landmarks. The third objective was to understand the evolutionary history of rDNA loci of *Gossypium*.

Materials and methods

Probe DNA isolation and labeling. DNA of pAm033 (a 470 bp BamHI fragment of the 5S rDNA repeat of *Acacia melanoxylon* in pUC118, kindly provided by Rudi Appels) and pGMR3 (a 4.5 kb EcoRI fragment of the 18S-28S ribosomal repeat of *Glycine max* in pBR325, kindly provided by Elizabeth Zimmer) was isolated by alkaline lysis in plasmid maxipreps as described by Silhavy et al. (1984). Total genomic DNA was isolated from immature leaves of *G. raimondii* ($2n=2x=26=D_5D_5$) and *G. arboreum* ($2n=2x=26=A_2A_2$) (accessions given below) using the technique described by Paterson et al. (1993). Whole plasmid and total genomic DNAs were labeled with biotin-14-dATP (Gibco BRL) using the Gibco BRL BioNick Labeling System or with digoxigenin-11-dUTP (Boehringer-Mannheim) using the Boehringer-Mannheim nick-translation kit.

Plant material, pretreatment and metaphase preparation. Root tips of *G. hirsutum* ($2n=4x=52=[(AD_1)]^2$) (Deltapine 50, Delta and Pine Land Co.), *G. arboreum* (Accession no. A2-92) and *G. herbaceum* ($2n=2x=26=A_1A_1$) (Accession no. unknown) were clipped into 1–2 cm lengths and pretreated for 4 h with 2.5 mM 8-hydroxyquinoline (aq) at room temperature. Root tips of *G. raimondii* (Accession no. D5-1 A22) and *G. thurberi* ($2n=2x=26=D_1D_1$) (Accession no. D1-1) were clipped into 2–3 cm lengths and pretreated with 2.5 mM 8-hydroxyquinoline (aq) at room temperature for 5 h. Following pretreatment, root tips were fixed overnight in 4:1 ethanol:acetic acid at room temperature. All accessions were obtained from the National Collection of *Gossypium* Germplasm, College Station, Texas. Metaphase spreads were prepared as described by Jewell and Islam-Faridi (1994).

In situ hybridization. The procedure used was a modification of that of Islam-Faridi and Mujeeb-Kazi (1995). Slides were immersed in 30 µg/ml RNase in 2×SSC for 45 min at 37°C, denatured at 70°C in 70% formamide, 2×SSC for 2.5 min and dehydrated in 70%, 85%, 95% and 100% ethanol for 2 min each at –20°C.(1×SSC is 0.15 M NaCl, 0.015 M sodium citrate). The probe mix was denatured at 80°C for 5 min, applied to the air-dried slide in a 25 µl volume, covered with a 20 mm×40 mm coverslip and sealed with rubber cement. The slides were then put in a humidity chamber in an 80°C oven for 7 min and allowed to incubate overnight at 37°C. Each 25 µl of hybridization mixture contained 50% deionized formamide, 10% dextran sulfate, 9 µg *Escherichia coli* DNA, and 90 ng of biotin-labeled probe DNA in 2×SSC. For dual labeling, 90 ng of biotin-labeled 18S-28S probe and 90 ng of digoxigenin-labeled 5S rDNA probe were used per slide. For genomic "painting" of the A-subgenome 50 ng of biotin-labeled total genomic DNA of *G. arboreum*, 1 µg unlabelled genomic DNA of *G. raimondii* and 8 µg *E. coli* DNA were used per slide.

Following overnight incubation at 37°C, coverslips were removed and slides were rinsed at 40°C in: 2×SSC, 5 min; 2×SSC, 5 min; 2×SSC, 50% formamide, 10 min; 2×SSC, 5 min; 2×SSC, 5 min; and 4×SSC, 5 min. Signal from biotin-labeled probes was then amplified with sequential 30 min applications of 5 µg/ml fluorescein isothiocyanate (FITC)-avidin DCS (Vector) in 4×SSC, 0.2% Tween 20, 10 µg/ml biotinylated anti-avidin D (Vector) in 4×SSC, 0.2% Tween 20 and 5 µg/ml FITC-avidin in 4×SSC, 0.2% Tween 20. For two-probe detection, a final incubation with 20 µg/ml rhodamine anti-digoxigenin (Boehringer Mannheim) was added. After each step three washes were performed in 4×SSC, 0.2% Tween 20 for 5 min each at 37°C. Slides were stained in 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in McIlvaines buffer (9 mM citric acid, 80 mM $Na_2HPO_4 \cdot H_20$, 2.5 mM $MgCl_2$) for 20 min at room temperature (RT), then with propidium iodide (PI; 20 µg/ml in 2×SSC) for 30 min at RT, and finally, antifade was applied under a 22 mm×40 mm coverslip. For two-probe detection, no PI was used. After FISH detection of 18S-28S rDNA loci of *G. hirsutum* and photography of selected metaphase spreads was complete, coverslips were soaked off in 2×SSC, allowed to destain in 2×SSC for 30 min, dehydrated in a 70%, 85% and 95% ethanol series at RT, and air-dried. Genomic painting was then carried out on the slides as described above.

Microscopy. Images were photographed directly on Fuji ASA 400 film with Olympus AX-70 (Figs. 1A, B, D, E, 3B, C, E, 4B–D), Olympus Vanox (Figs. 1C, 3A, D), Zeiss Axioscope (Fig. 2A) and Zeiss Axiophot (Fig. 4A) epifluorescence microscopes using standard filter sets for DAPI, FITC and FITC/rhodamine/DAPI excitation.

Results

To detect 18S-28S rDNA loci with the greatest possible sensitivity, single-label FISH was used rather than dual-label FISH. The description and number of 18S-28S rDNA loci we detected using this method are given in Table 1; photomicrographs of the results are shown in Fig. 1A–E. For simplicity, 18S-28S rDNA FISH sites

Table 1. The distribution of 18S-28S rDNA loci in *Gossypium hirsutum* and closely related diploid species

Haploid genome or subgenome	Major 18S-28S rDNA loci	Intermediate 18S-28S rDNA loci	Minor 18S-28S rDNA loci	Total
G. hirsutum ([AD]1)	3	1	7	11
(A)	1	1	3	5
(D)	2	0	4	6
G. herbaceum (A1)	3	0	0	3
G. arboreum (A2)	2	0	1	3
G. raimondii (D5)	2	1	1	4
G. thurberi (D1)	4	0	0	4

Headings A and D represent the A- and D-subgenomes of *G. hirsutum*, as determined by genomic painting with labeled total A-genome (*G. arboreum*) DNA

were described as follows. Major sites were defined as those giving very large signals observable in all interphase and metaphase cells. Much smaller FISH signals, which were nevertheless detected with high frequency in interphase and metaphase cells, were described as intermediate sites. The smallest FISH signals, which were generally detectable in at least 40% of metaphase cells, were described as minor sites. Based on results from FISH of bacterial artificial chromosome clones of *G. hirsutum* (Hanson et al. 1995), we estimate that the smallest rDNA signals reported herein represent loci in the 25–35 kb size range.

The four largest 18S-28S rDNA loci of *G. hirsutum* were previously mapped by molecular-meiotic configuration analysis to chromosomes 7, 9, 16 and 23 (Price et al. 1990; Crane et al. 1993). A smaller fifth site was observed but not mapped. Using somatic metaphase preparations and improved protocols for in situ hybridization (ISH) and detection, we were able consistently to detect an additional six minor sites, bringing the total reported 18S-28S rDNA loci to 11 (Fig. 1E). Similar results have also been obtained in our laboratory using meiotic chromosomes. To avoid confusion of signals with background noise, which was extremely low, only loci at which a pair of signals was visible, one per chromatid, were scored. Eleven loci were observable in greater than 30% of the approximately 70 *G. hirsutum* metaphase spreads we examined. A minimum of 30 metaphase spreads was examined for enumeration of diploid 18S-28S rDNA loci.

To determine the subgenomic locations, A versus D, of 18S-28S loci in the *G. hirsutum* genome, metaphase spreads that had been used for FISH with the 18S-28S rDNA probe were destained, dehydrated in an ethanol series, air-dried and then reprobed with labeled A2-genome DNA (Fig. 2). It was found that addition of a 20-fold excess of unlabeled total genomic DNA of *G. raimondii* to the probe mix was sufficient to suppress cross-hybridization of sequences that are shared between the two genomes, with the exception of major loci of the highly conserved 18S-28S rDNA repeat. Results show that five 18S-28S loci can be localized to the *G. hirsutum* A-subgenome and six to the D-subgenome (Table 1). The number of 18S-28S loci in the A-subgenome is two more than the additive total observed in each A-diploid, and the number of 18S-28S loci in the D-subgenome is two more than was observed in each of the D-diploids (see Table 1). We observed no chromosomes with painted and unpainted segments, other than the 18S-28S rDNA loci bearing D-subgenome chromosomes, which suggests that no large intergenomic translocations have occurred following polyploidization. This conclusion was supported by genomic painting results using D-diploid DNA as a probe on other metaphase spreads of *G. hirsutum* chromosomes (not shown).

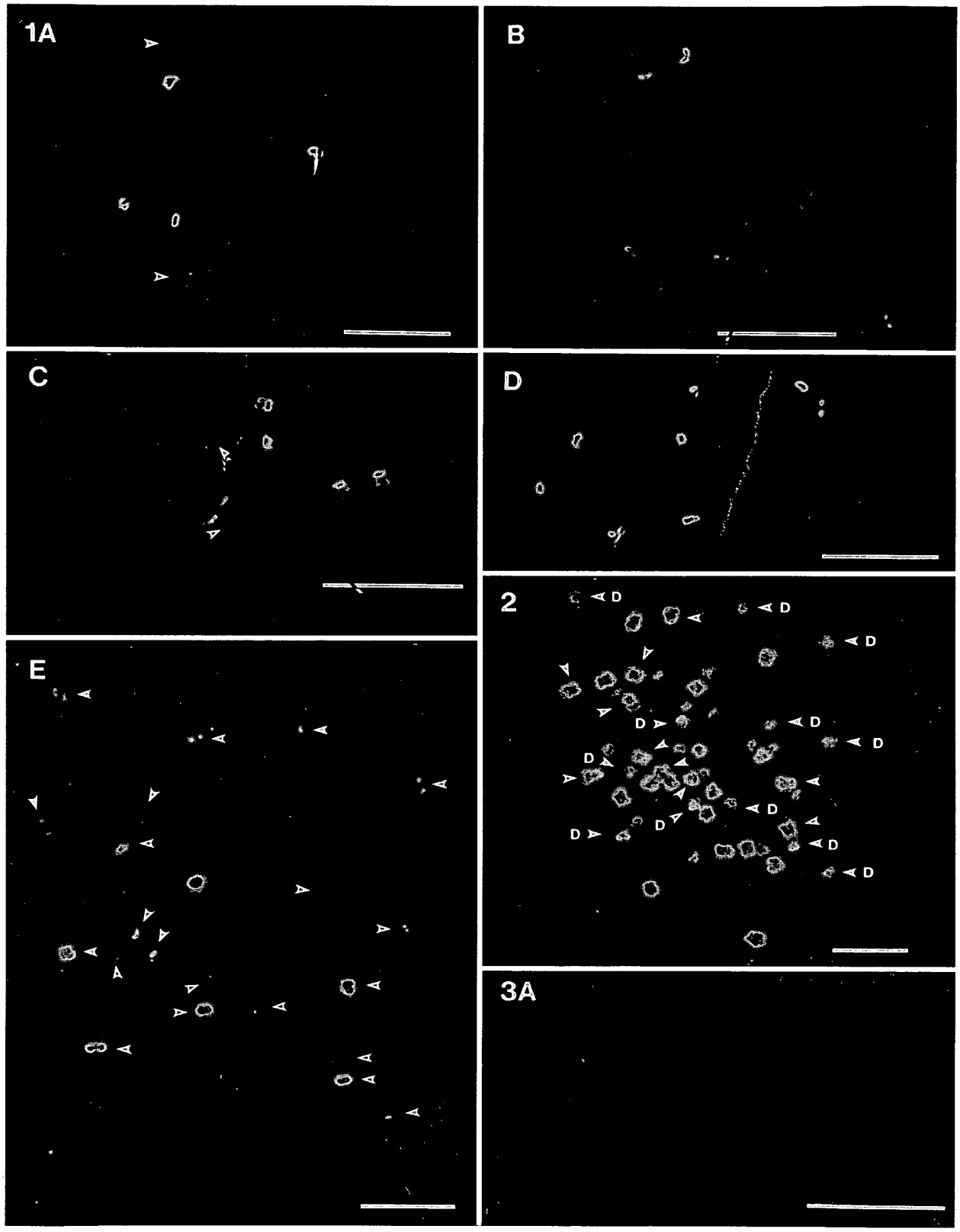
In all the diploids examined, only a single major pair of 5S rDNA FISH signals was observed per haploid genome, located near the centromere on the short arm of the respective chromosome (Fig. 3A–D). Two pairs of signals of unequal size were detected in *G. hirsutum*. The larger 5S rDNA locus was in the long arm near the

Fig. 1A–E. Fluorescence photomicrographs of cotton metaphase chromosomes hybridized with a biotin-labeled 18S-28S rDNA probe. Signals were detected with fluorescein isothiocyanate (FITC)-conjugated avidin and chromosomes were counterstained with propidium iodide (PI). **A** Partial metaphase from *G. arboreum* (A_1), showing all three 18S-28S loci. Two major loci and one minor site (*arrowheads*) are visible. **B** *G. herbaceum* (A_2); three major loci are visible. **C** *G. raimondii* (D_5) shows two major sites, an intermediate site, and one minor site (*arrowheads*). The minor site is extremely small and is located near the centromere on the short arm of the chromosome. **D** *G. thurberi* (D_1); four major loci are visible. **E** *G. hirsutum* ($[AD]_1$); *arrowheads* indicate the 11 observable loci. Three loci are major sites, one is an intermediate site and seven are minor. Bars represent 10 μm

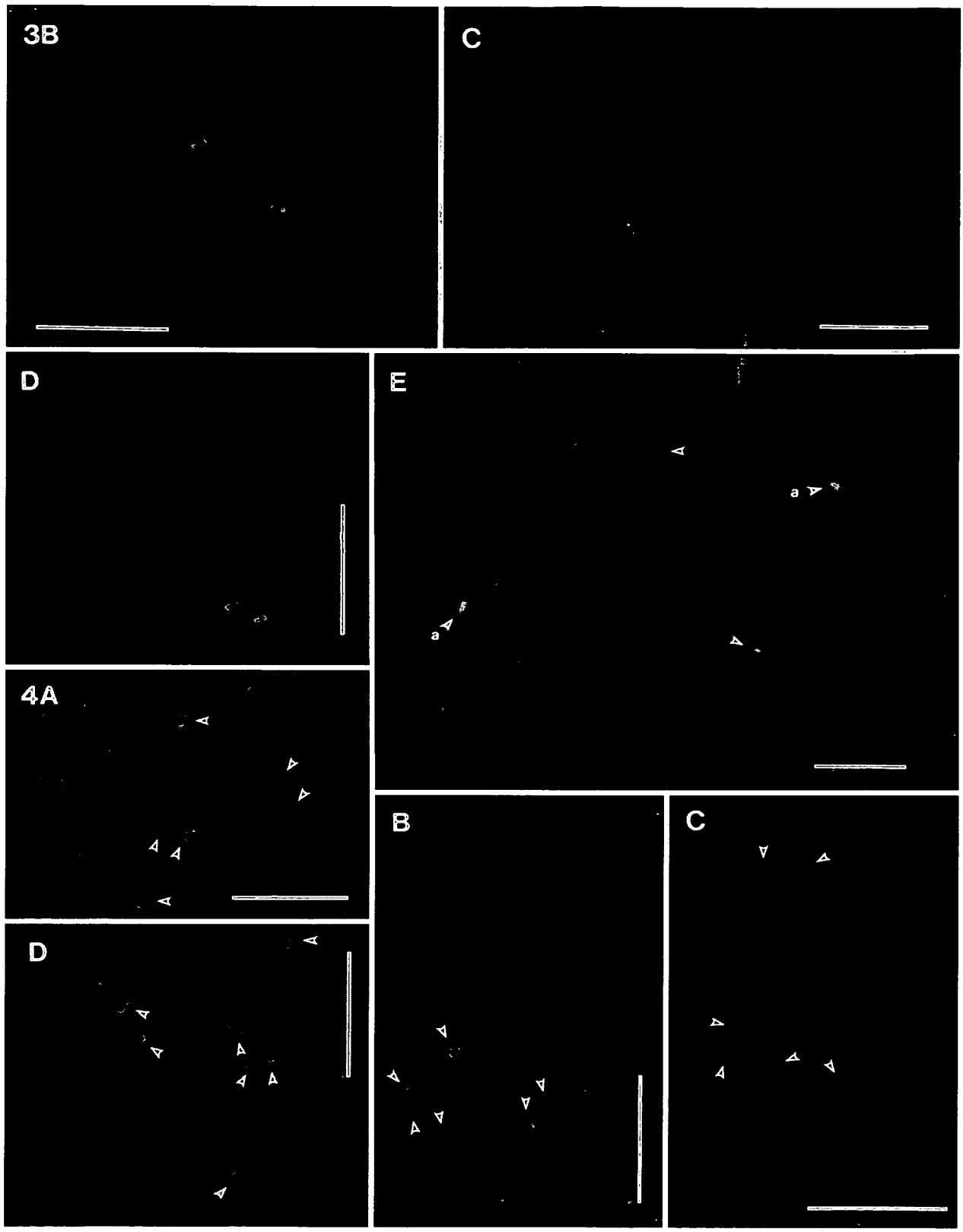
Fig. 2. Fluorescence photomicrograph of the metaphase spread of *G. hirsutum* shown in Fig. 1E rehybridized with biotin-labeled total A-genome (*G. arboreum*) DNA. Signal was detected with FITC-conjugated avidin and chromosomes were counterstained with PI. Unlabeled total D-genome (*G. raimondii*) DNA was used in a 20-fold excess to block cross-genomic hybridization. *Arrowheads* show chromosomes that bear 18S-28S rDNA loci; *arrowheads labeled D* represent chromosomes from the D-subgenome and *unlabeled arrowheads* represent chromosomes from the A-subgenome. The strong signals on two pairs of D-subgenome chromosomes are due to hybridization of the highly conserved 18S-28S rDNA repeat. No other areas of "painted" and "unpainted" segments can be observed on A- or D-subgenome chromosomes. Bar represents 10 μm

Fig. 3A–E. Fluorescence photomicrographs of cotton metaphase chromosomes hybridized with a biotin-labeled 5S rDNA probe. Signal was detected with FITC-conjugated avidin and chromosomes were counterstained with PI. In all the diploid species (**A–D**), one locus is visible, located near the centromere on the short arm of a homologous pair of chromosomes. **A** *G. thurberi* (D_1); note the PI staining of major 18S-28S rDNA loci. **B** *G. arboreum* (A_2). **C** *G. herbaceum* (A_1). **D** *G. raimondii* (D_5). **E** *G. hirsutum* ($[AD]_1$); two loci are visible. The larger pair of signals (*arrowheads labeled a*) are located on the long arm of chromosome 9 and the smaller pair of signals (*unlabeled arrowheads*) are located on the short arm of chromosome 23. Propidium iodide staining allows for the visualization of major 18S-28S rDNA loci syntenic to both 5S rDNA loci, on the short arm of the chromosome. Bars represent 10 μm

Fig. 4A–D. Fluorescence photomicrographs of metaphase chromosomes of diploid cotton simultaneously tested with a biotin-labeled 18S-28S probe and a digoxigenin-labeled 5S rDNA probe. The 18S-28S rDNA probe was detected with FITC-conjugated avidin (*yellow/green*) and the 5S rDNA probe was detected with rhodamine-conjugated anti-digoxigenin antibodies (*red*). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI). *Arrowheads* indicate 18S-28S rDNA loci. In each figure the red-colored 5S rDNA signals are clearly visible. **A** *G. arboreum* (A_2). Three 18S-28S loci are visible; the 5S site is syntenic to the minor 18S-28S site, which can very faintly be seen on the short arm of the chromosome. **B** *G. herbaceum* (A_1). All three major 18S-28S loci are clearly visible; the 5S site is syntenic to one of the two telomerically located major loci. **C** *G. raimondii* (D_5). Three 18S-28S loci can be seen; the 5S site is syntenic to an intermediate 18S-28S site. **D** *G. thurberi*(D_1) shows the four major 18S-28S rDNA loci; signal is not visible in the photograph for one chromosome. The 5S site is syntenic to a major 18S-28S site. Bars represent 10 μm



Figs. 1A–3A (for legends see p. 57)



Figs. 3B–4D (for legends see p. 57)

centromere of a medium-sized pair of A-subgenome chromosomes, whereas the smaller locus was in the short arm near the centromere of a small- to medium-sized pair of D-subgenome chromosomes (Fig. 3E).

Differentially bright PI fluorescence allowed for the simultaneous visualization of major 18S-28S rDNA loci (red) and FITC-avidin-detected 5S rDNA loci (yellow/green) in *G. hirsutum*. Major 18S-28S rDNA loci were observed to be syntenic to each of the two 5S rDNA loci of *G. hirsutum*, corroborating the positions established by Crane et al. (1993) using molecular meiotic ISH (Fig. 3E). In the diploid species, the syntenic relationships of the 5S rDNA site relative to the 18S-28S rDNA site were determined by simultaneous detection of a biotin-labeled 18S-28S rDNA probe and a digoxigenin-labeled 5S rDNA probe (Fig. 4A–D). The results revealed that the 5S rDNA loci of both *G. herbaceum* (A1) and *G. thurberi* (D1) were syntenic to the major 18S-28S rDNA loci (Fig. 4B, D); whereas those of *G. arboreum* (A_2) *and G. raimondii* (D_5) were syntenic to minor and intermediate 18S-28S rDNA loci, respectively, and not to major 18S-28S rDNA loci, as would be expected based on the *G. hirsutum* data (Fig. 4A, C).

Discussion

A number of hypotheses may explain why all 18S-28S rDNA loci of *G. hirsutum* were not accounted for in diploid progenitors. First, the number of copies of the rDNA repeat present at some A- and D-diploid loci may be too few to be detected. Hence, a discrepancy in the number of detectable 18S-28S rDNA loci could be explained either by elimination of copies of the 18S-28S repeat in modern A- and D-diploids relative to the ancestral genome donors, or by post-polyploidization amplification of those 18S-28S rDNA loci of the tetraploid that originally bore few repeats. Given an estimated limit of 25–35 kb for consistent signal detection, and the length of the probe used (4.5 kb), we infer that our current threshold for detection of 18S-28S rDNA repeats is approximately five to eight copies. A second possible explanation is that translocations with breakpoints located in the middle of 18S-28S rDNA loci may have bisected and thus duplicated sites following polyploidization of *G. hirsutum*. Third, increases and/or decreases in site number could arise by the formation of translocations with breakpoints proximal to the rDNA sites and subsequent fixation of duplication-deficiencies. Fourth, new rDNA loci may have been formed in the tetraploid by transposition of sequences containing rDNA repeats. Fifth, deletions may have eliminated loci in modern A- and D-diploids. And finally, the diploids examined may not represent the ancestral donors of the tetraploid A- or D-subgenomes.

The two 5S rDNA loci of *G. hirsutum* were previously mapped using ISH. A large site of 5S rDNA ISH was mapped to the long arm of chromosome 9 and a somewhat smaller site was localized to the short arm of chromosome 23 (Crane et al. 1993). Based on the differences in chromosomal arm location of 5S rDNA loci, Crane et al. (1993) proposed that following divergence of the A- and D-genomes, either a pericentric inversion or a transposition had occurred in the homolog of chromosome 9 or chromosome 23. Our data, showing 5S rDNA loci located near the centromere in the short arm of all A- and D-diploids observed (Fig. 3A–D), suggest that the rearrangement occurred on chromosome 9 and that this occurred after the polyploidization event leading to *G. hirsutum*. This conclusion is concordant with independent restriction fragment length polymorphism (RFLP) mapping data (Reinisch et al. 1994; J. F. Wendel, personal communication).

The results from simultaneous FISH of 5S and 18S-28S rDNA probes to chromosomes of *G. herbaceum* (A_1) and *G. thurberi* (D_1) were concordant with results from the tetraploid in that the 5S rDNA sites were syntenic to major 18S-28S rDNA loci (Fig. 4B, D). The results for *G. arboreum* (A_2) and *G. raimondii* (D_5) were not as predicted, however, as neither of the 5S sites was syntenic with a major 18S-28S rDNA locus (Fig. 4A, C). The *G. raimondii* data are particularly interesting, as it has been reported that this species is the most likely D-genome ancestor of the extant tetraploids (Endrizzi et al. 1985). If this is indeed the case, then our results indicate that, barring reciprocal rearrangement between minor and major rDNA loci, the 18S-28S rDNA cluster of *G. hirsutum* on the D-subgenome chromosome 23 has been significantly amplified in size following polyploidization, or that this same locus has been significantly deamplified in modern *G. raimondii* lineages.

The size distribution of the 18S-28S rDNA sites observed in the diploids was not as expected. Based on our genomic painting data from *G. hirsutum* and the previously mentioned ISH mapping data (Crane et al. 1993), the expected pattern in the A-genome diploid species included one major site corresponding to chromosome 9, one intermediate site corresponding to chromosome 7, and three minor sites. The expected pattern of the D-genome species included two major 18S-28S rDNA sites corresponding to chromosomes 16 and 23, and four minor sites. In A-genome diploids no fewer than two major 18S-28S rDNA sites were observed, suggesting that at least one major site was significantly deamplified in the A-subgenome of the tetraploid following polyploidization, or that at least one site has been greatly amplified in modern diploid ancestors of the A-subgenome donor. In *G. raimondii* the number of major 18S-28S loci observed was as expected, although neither of the two major 18S-28S rDNA loci we observed was located on the predicted chromosome 23 homolog, which bears a 5S site. It is therefore suggested, that one of these two major 18S-28S sites in *G. raimondii* is homologous to the major site on chromosome 16 of *G. hirsutum*, while the other has either been significantly amplified in *G. raimondii* relative to the ancient D-subgenome donor or greatly deamplified in the tetraploid following polyploidization.

Several mechanisms may account for the changes in sizes of rDNA loci that we observed. One proposed way that tandem repeats such as rDNA loci can be significantly amplified or deleted is through both homologous and nonhomologous unequal crossing over (Arnheim et al. 1980; Seperack et al. 1988). Anderson and Roth

(1981) have shown that in *Salmonella typhimurium* unequal crossing over takes place much more frequently at rDNA loci than at 37 other loci observed. Recent data obtained by Wendel et al. (1995) have demonstrated the occurrence of interlocus concerted evolution among rDNA loci in *Gossypium* tetraploids following polyploidization, which may suggest that unequal crossing over takes place between nonhomologous rDNA loci in *G. hirsutum*. The fact that rDNA-bearing chromosomes appear to be nonrandomly associated with each other at mitotic metaphase (Figs. 1A–D, 3B–C, 4A–C) may support the theory that somatic exchange takes place between nonhomologous loci, in which case homogenization and changes in rDNA copy number could occur especially quickly, particularly in plants and other organisms without germ lines that are differentiated early in development. In rye, it has been shown that the telomeres of some "associated" chromosomes are more alike in their arrays of repeats than those of "un-associated" chromosomes (Bennett 1982). Significant to this potential mechanism is that 12 of 14 18S-28S rDNA loci observed in the diploid species and all 3 major 18S-28S loci of *G. hirsutum* are located at either telomeric or slightly subtelomeric positions, as this would possibly allow significant rearrangements to occur between rDNA loci without deleterious effects to the cell. It therefore seems reasonable to propose that in species with mostly telomerically located 18S-28S rDNA repeats there will be observed on average: a greater degree of interlocus concerted evolution, a higher number of loci, and more variability in locus number and size, both within and between closely related species.

The multiplicity of sites, facility of detection and relative variability of rDNA loci indicate that rDNA loci will have considerable utility in future molecular cytogenetic studies of *G. hirsutum* and its diploid relatives. Further, the data indicate that rDNA loci have much to offer as a tool for analyzing the evolution and behavior of tandemly repeated gene families, how they contribute to genomic elasticity, and how they may promote or constrain changes relevant to polyploidization. Subsequent studies using FISH of both repetitive and single-copy sequences will likely add to the information we have presented and contribute greatly to our understanding of the homologous relationships between the chromosomes of *G. hirsutum* and the chromosomes of A- and D-diploids, and more generally, to genome evolution per se.

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References

Anderson P, Roth R (1981) Spontaneous tandem genetic duplications in *Salmonella typhimurium* arise by unequal recombination between rRNA cistrons. Proc Natl Acad Sci USA 78: 3113–3117

Arnheim N, Krystal M, Schmickel R, Wilson G, Ryder O, Zimmer E (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and ape. Proc Natl Acad Sci USA 77: 7323–7327

Beasley JO (1940) The origin of American tetraploid *Gossypium* species. Am Nat 74: 285–286

Beasley JO (1942) Meiotic chromosome behavior in species, species hybrids, haploids and induced polyploids of *Gossypium*. Genetics 27: 25–54

Bennett MD (1982) Nucleotypic basis of the spatial ordering of chromosomes in eukaryotes and the implications of the order for genome evolution and phenotypic variation. In: Dover GA, Flavell RB (eds) Genome evolution. Academic Press, London, pp 239–262

Crane CF, Price HJ, Stelly DM, Czeschin DG, McKnight TD (1993) Identification of a homeologous chromosome pair by *situ* hybridization to ribosomal RNA loci in meiotic chromosomes of cotton (*Gossypium hirsutum* L.). Genome 36: 1015–1022

Edwards GA, Mirza MA (1979) The designation of a new G genome for *Gossypium bickii*. Can J Genet Cytol 21: 367–372

Endrizzi JE, Turcotte EL, Kohel RJ (1985) Genetics, cytology, and evolution of *Gossypium*. Adv Genet 23: 271–375

Fryxell PA (1992) A revised taxanomic interpretation of *Gossypium* L. Rheedea 2: 108–165

Hanson RE, Zwick MS, Choi SD, Islam Faridi MN, McKnight TD, Wing RA, Price HJ, Stelly DM (1995) Fluorescent in situ hybridization of a bacterial artificial chromosome. Genome 38: 646–651

Islam-Faridi MN, Mujeeb-Kazi A (1995) *Secale cereale* DNA in wheat germplasm; visualization by fluorescent in situ hybridization and breeding application. Theor Appl Genet 90: 595–600

Jewell DC, Islam-Faridi MN (1994) Details of a technique for somatic chromosome preparation and C-banding of maize. In: Freeling M, Walbot V (eds) The maize handbook. Springer, Berlin Heidelberg New York, pp 484–493

Menzel MY (1954) A cytological method for genome analysis in *Gossypium*. Genetics 40: 214–223

Paterson AH, Brubaker KL, Wendel JF (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol Biol Rep 11: 122–127

Price HJ, Stelly DM, McKnight TD, Scheuring CF, Raska D, Michaelson MJ, Bergey D (1990) Molecular cytogenetic mapping of a nucleolar organizer region in cotton. J Hered 81: 365–370

Reinisch AJ, Dong J, Brubaker CL, Stelly DM, Wendel JF, Paterson AH (1994) A detailed RFLP map of cotton, *Gossypium hirsutum* × *Gossypium barbadense*: chromosome organization and evolution in a disomic polyploid genome. Genetics 138: 829–847

Seperack P, Slatkin M, Arnheim N (1988) Linkage disequilibrium in human ribosomal genes; implications for multigene family evolution. Genetics 119: 943–949

Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with gene fusion. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 144–146

Skovsted A (1934) Cytological studies in cotton. II. two interspecific hybrids between Asiatic and New World cottons. J Genet 28: 407–424

Wendel JF (1989) New World tetraploid cottons contain Old World cytoplasm. Proc Natl Acad Sci USA 86: 4132–4136

Wendel JF, Albert VA (1992) Phylogenetics of the cotton genus (*Gossypium* L.): character-state weighted parsimony analysis of chloroplast DNA restriction site data and its systematic and biogeographic implications. Syst Bot 17: 115–143

Wendel JF, Schnabel A, Seelanan T (1995) Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc Natl Acad Sci USA 92: 280–284

Sequential chromosome banding and in situ hybridization analysis[1]

JIMING JIANG AND BIKRAM S. GILL[2]

Wheat Genetics Resource Center and Department of Plant Pathology, Kansas State University, Manhattan, KS 66506-5502, U.S.A.

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Different combinations of chromosome N- or C-banding with in situ hybridization (ISH) or genomic in situ hybridization (GISH) were sequentially performed on metaphase chromosomes of wheat. A modified N-banding–ISH/GISH sequential procedure gave best results. Similarly, a modified C-banding – ISH/GISH procedure also gave satisfactory results. The variation of the hot acid treatment in the standard chromosome N- or C-banding procedures was the major factor affecting the resolution of the subsequent ISH and GISH. By the sequential chromosome banding – ISH/GISH analysis, multicopy DNA sequences and the breakpoints of wheat–alien translocations were directly allocated to specific chromosomes of wheat. The sequential chromosome banding– ISH/GISH technique should be widely applicable in genome mapping, especially in cytogenetic and molecular mapping of heterochromatic and euchromatic regions of plant and animal chromosomes.

Key words: N-banding, C-banding, in situ hybridization, genomic in situ hybridization.

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Différentes combinaisons de révélation des bandes C ou N par hybridation in situ (ISH) des chromosomes ou par hybridation in situ des génomes (GISH) ont été expérimentées de façon sériée sur des chromosomes de blé en métaphase. Une méthode sériée modifiée de révélation des bandes N par ISH/GISH a donné les meilleurs résultats. De façon similaire, une méthode sériée de révélation des bandes C par ISH/GISH a également donné des résultats satisfaisants. La variation du traitement à l'acide chaud dans les méthodes standards de révélation des bandes C et N des chromosomes a été le facteur majeur qui a affecté la résolution des traitements ISH/GISH subséquents. Par analyses des ISH/GISH révélant de façon sériée les bandes chromosomiques, les séquences d'ADN en multicopies et les points de fracture « blé-translocation étrangère » ont été directement assignés à des chromosomes spécifiques du blé. La technique de révélation sériée ISH/GISH des bandes chromosomiques devrait être largement applicable pour la cartographie des génomes et, plus spécifiquement, la cartographie cytogénétique et moléculaire des régions hétérochromatiques et euchromatiques des chromosomes des plantes et des animaux.

Mots clés : bandes N, bandes C, hybridation in situ, hybridation in situ des génomes.

[Traduit par la rédaction]

Introduction

Chromosome banding and in situ hybridization are two pivotal techniques for cytogenetics. Identification of individual chromosomes of human and most of the animal and plant species was impossible until the development of various chromosome banding techniques (for review see Hsu 1973). In situ hybridization is not only the most direct method for physical mapping of DNA sequences on chromosomes but also an alternative method for chromosome identification with different DNA markers.

In humans, chromosome banding and in situ hybridization techniques were successfully integrated into a single procedure. DNA sequences can be directly and more precisely mapped to a specific chromosome area by combining these techniques (see review by McNeil et al. 1991). Although both chromosome banding and in situ hybridization have been successfully applied for cytogenetic studies in plants, especially in wheat (*Triticum aestivum*) and its related species, reports on the sequential use of the two techniques have been limited (Hutchinson and Seal 1983). Thus, the potential of developing sequential banding – in situ hybridization techniques for plant chromosome analysis has not been fully exploited. Here, we report on the results of sequential analysis on wheat metaphase chromosomes using different combinations of N- or C-banding with in situ hybridization (ISH) or genomic in situ hybridization (GISH).

Materials and methods

Wheat variety 'Chinese Spring' (CS) and a wheat germplasm line KS92WGRC19 containing wheat–rye (*Secale cereale*) translocations T1AL·1RS and T4BS·4BL–6RL (Friebe et al. 1991) were used as the test materials.

The chromosome preparation and N-banding technique were according to Endo and Gill (1984). To integrate the N-banding technique with in situ hybridization, the major

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[2]Author to whom all correspondence and reprint requests should be addressed.

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modification was the omission of the hot 45% acetic acid treatment. After removing the cover slips, the slides were dried in an ethanol series (75, 95, and 100% ethanol, 5 min each), then directly incubated in hot phosphate buffer (1 M NaH_2PO_4) at 92 ± 2°C for 2 min, rinsed with tap water, and immediately stained with a Banco Giemsa solution (dilute one drop of stock solution with 1 mL of 1/15 M Sørenson's phosphate buffer) for 22–24 min. After recording cells with well-spread N-banded metaphase chromosomes on film, the slides were destained in an ethanol series (75, 95, and 100% ethanol, 5 min each) and were processed for in situ hybridization. The same cells were rephotographed after in situ hybridization and the results were compared.

The C-banding technique was as described (Gill and Kimber 1974; for review see Gill et al. 1991). The modification of the C-banding procedure for sequential C-banding – in situ hybridization analysis included varying the duration and temperature of the 0.2 M HCl treatment. In addition to the standard treatment of chromosome preparations in 0.2 M HCl at 60°C for 2 min, treatment at 37°C and room temperature for 10–40 min were applied. After taking photos of the well-spread C-banded metaphase chromosome figures, the slides were destained in an ethanol series and were processed for in situ hybridization. The same metaphase chromosome figures were rephotographed after in situ hybridization and the results were compared.

ISH and diaminobenzidine tetrahydrochloride (DAB) detection techniques were according to Rayburn and Gill (1985). GISH and fluorescence detection techniques were as described (Le et al. 1989; Mukai and Gill 1991; Friebe et al. 1993). A ribosomal gene clone was used as a probe in ISH. This probe contains a single wheat 18S·26S rRNA gene repeat unit that originated from plasmid pTa71 (Gerlach and Bedbrook 1979).

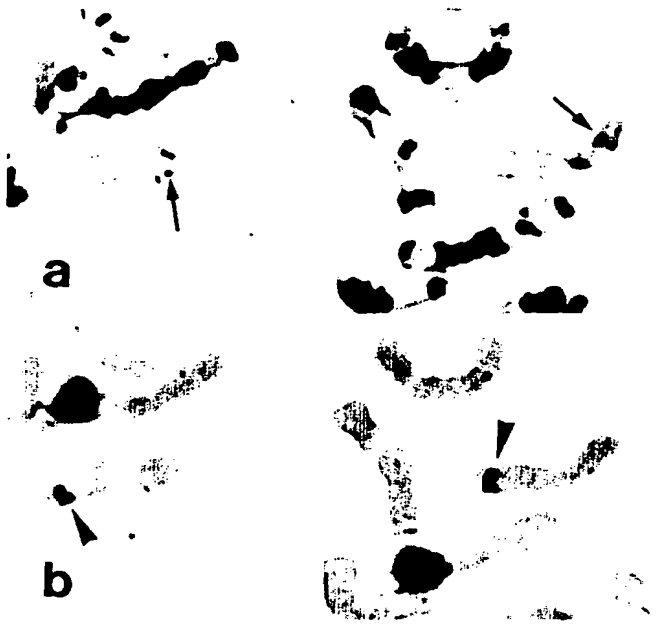

FIG. 1. Sequential modified N-banding – ISH (using 18S·26S ribosomal genes as a probe) analysis of 'Chinese Spring' (CS) wheat. (*a*) A part of a cell of CS with the modified N-banding. (*b*) The same cell after ISH. The N-band (arrow) on the short arm is diagnostic for the identification of this chromosome as 7D. The subsequent ISH locates an rRNA locus on the long arm of 7D (arrowhead).

Results

We tried both ISH/GISH – N-banding and N-/C-banding – ISH/GISH sequential combinations. The results of the sequential ISH/GISH – N-banding experiments showed that (*i*) the quality of N-banding following ISH with different probes was always poor; (*ii*) it was difficult to remove the hybridization signal detected with the DAB method (the banding result was better after fluorescence than after DAB detection); and (*iii*) it was impossible to get good banding following GISH.

The experiments involving sequential N-/C-banding – ISH/GISH procedures were much more successful. Despite some loss of resolution, both ISH and GISH were successfully performed after the standard N-banding. The resolution was improved by deleting the hot acetic acid treatment used in the standard N-banding procedure. Although the quality of the bands using the modified N-banding technique was usually not as good as that of the standard technique, all the N-bands on 16 pairs of CS wheat were consistently observed (Endo and Gill 1984). The smallest 18S·26S ribosomal gene cluster on chromosome 7D of CS (see Mukai et al. 1991) was localized with the modified N-banding – ISH procedure, indicating little loss of the ISH resolution (Fig. 1).

Figures 2*a* and 2*b* show a part of a cell of KS92WGRC19 by the procedure of modified N-banding – GISH using genomic DNA of rye as a probe. T1AL·1RS had no N-bands. T4BS·4BL–6RL showed a large N-band near the centromere on both arms. The breakpoints on both translocation chromosomes were clearly localized by the subsequent GISH.

Standard C-banding – ISH/GISH combinations reduced the resolution of the ISH/GISH compared with N-banding – ISH/GISH combinations. The resolution of ISH and GISH was improved by the use of a modified C-banding procedure. In one experiment, we found that if the chromosome preparations were incubated in 0.2 M HCl at room temperature for 30 min, then the resolution of the GISH was better than with the standard HCl treatment. Most of the C-bands were visualized, although the quality of C-banding was decreased owing to the modification of the HCl treatment.

A part of a cell of KS92WGRC19 with modified C-banding – GISH procedure is shown in Figs. 2*c* and 2*d*. The rye-specific C-bands on 1RS of T1AL·1RS are clearly visible. Faint rye-specific C-bands on 6RL are also visible on T4BS·4BL–6RL. Although the contrast between the rye chromosome arm/segment and the wheat chromosomes in the subsequent GISH was not as good as that with the modified N-banding – GISH method, the location of breakpoints of the wheat–rye translocation chromosomes are still clear.

Discussion

Various combinations of chromosome banding techniques, i.e., G-, Q-, and replication banding, with in situ hybridization are routinely used in human chromosome analysis (see review by McNeil et al. 1991). Most of the combined techniques need separate photographic processes for the banding and in situ hybridization analyses. However, the techniques of simultaneous visualization of ISH signals and G-bands identified by bromodexyuridine (BrdU) incorporation are also available (Bhatt et al. 1988; Lawrence et al. 1990). These techniques have greatly facilitated

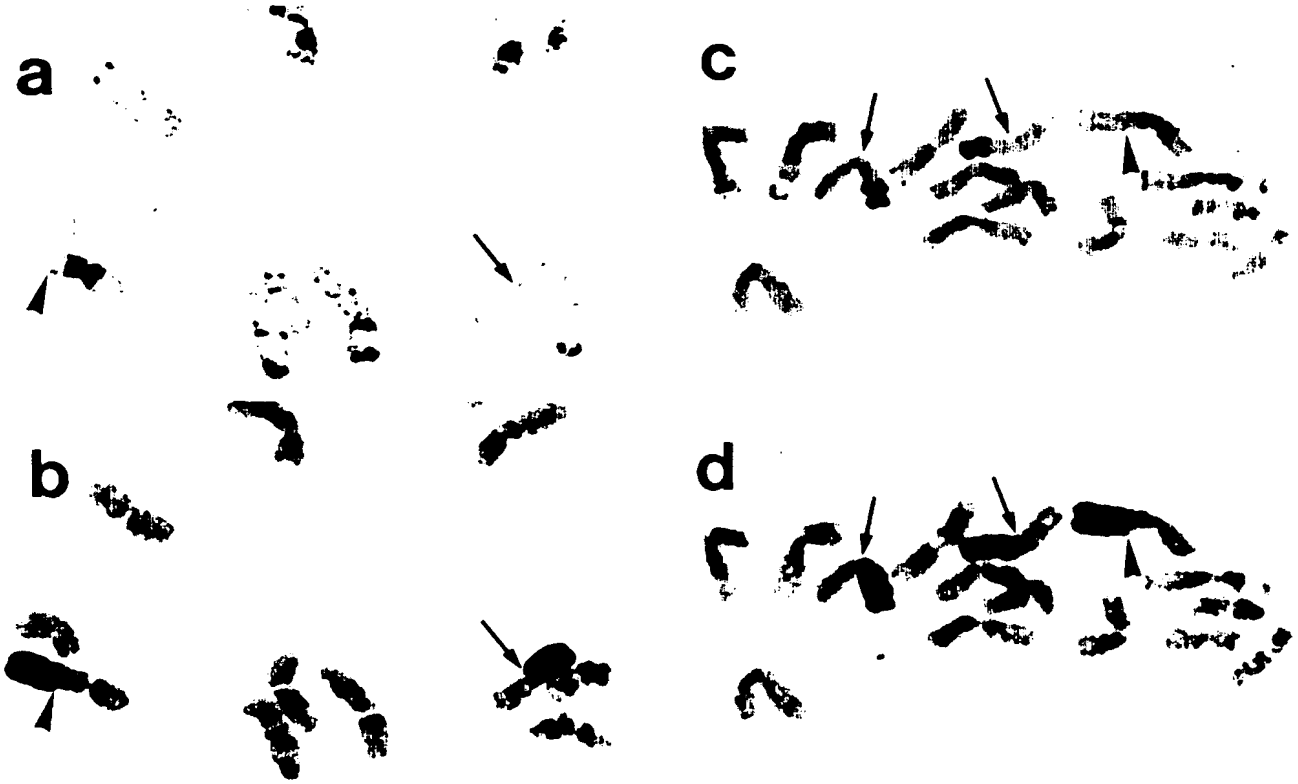

FIG. 2. Sequential modified N-/C-banding – GISH analysis of a wheat–rye translocation line KS92WGRC19. Arrows and arrowheads point to the breakpoints of the wheat–rye translocation chromosomes. Arrows, translocation chromosomes T1AL·1RS; arrowheads, translocation chromosomes T4BS·4BL–6RL. (*a*) A part of a cell of KS92WGRC19 with modified N-banding. (*b*) The same cell after GISH. (*c*) A part of a cell with modified C-banding (0.2 M HCl at room temperature for 30 min). (*d*) The same cell after GISH.

the physical mapping of human genes by ISH, as they allow chromosome identification and gene localization in one step.

The quality of banding after ISH may be variable and less optimal owing to morphological changes during chromosome denaturation (Lichter et al. 1991). The Giemsa–trypsin technique produces the highest resolution banding pattern for human chromosomes. However, trypsinization weakens the ISH signal (Lawrence et al. 1990). Thus, the application of this technique was limited. Replication banding and some other banding techniques, such as DAPI staining, do not interfere with ISH and are more widely used (McNeil et al. 1991).

In plants, sequential ISH (using radioactive probe) – C-banding analysis was reported on rye chromosomes (Hutchinson and Seal 1983). The resolution of the subsequent C-banding was reduced according to the authors' illustration. Sequential ISH (using biotin-labeled probes) – N-banding technique gave poor results in our hands. Furthermore, the results of GISH – N-banding were even more unsatisfactory.

Based on our results, the modified N-banding – ISH (using 18S·26S ribosomal genes as a probe) and modified N-banding – GISH gave excellent resolution. In fact, we consistently obtain better ISH and GISH contrast using the above procedures compared with standard ISH or GISH (without prior banding) using the same probes and same materials. It is not known why the contrast of ISH and GISH was improved after the modified N-banding. The hot acetic acid and HCl treatments in the regular N- and C-banding procedures are probably the major factors affecting the resolution of the subsequent ISH and GISH.

Although the modified N-banding – ISH/GISH gave the best results in the present study, unfortunately, not all the wheat chromosomes are marked with N-bands. In fact, the N-banding technique has been applied only to a limited number of plant species for chromosome identification, whereas C-banding is the most widely applicable banding technique in plants (Gill and Sears 1988). Therefore, we tried several modifications of the C-banding – ISH/GISH procedures. By modification of the 0.2 M HCl treatment in the present case, the modified C-banding–GISH technique showed sufficient resolution to locate the breakpoints and specific chromosomes in wheat–rye translocations. Therefore, the sequential C-banding – ISH technique should find wide application in cytogenetic and molecular analysis of chromosome structure and function.

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Bhatt, B., Burns, J., Flannery, D., and McGee, O'D. 1988. Direct visualization of single copy genes on banded meta-

phase chromosomes by nonisotopic *in situ* hybridization. Nucleic Acids Res. **16**: 3951–3961.

Endo, T.R., and Gill, B.S. 1984. Somatic karyotype, heterochromatin distribution, and nature of chromosome differentiation in common wheat, *Triticum aestivum* L. em Thell. Chromosoma, **89**: 361–369.

Friebe, B., Hatchett, J.H., Gill, B.S., Mukai, Y., and Sebesta, E.E. 1991. Transfer of Hessian fly resistance from rye to wheat via radiation-induced terminal and intercalary chromosomal translocations. Theor. Appl. Genet. **83**: 33–40.

Friebe, G., Jiang, J., Gill, B.S., and Dyck, P.L. 1993. Radiation-induced nonhomoeologous wheat – *Agropyron intermedium* chromosomal translocations conferring resistance to leaf rust. Theor. Appl. Genet. **86**: 141–149.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Gill, B.S., and Kimber, G. 1974. Giemsa C-banding and the evolution of wheat. Proc. Natl. Acad. Sci. U.S.A. **71**: 4086–4090.

Gill, B.S., and Sears, R.G. 1988. The current status of chromosome analysis in wheat. *In* Chromosome structure and function. *Edited by* J.P. Gustafson and R. Appels. Stadler Genet. Symp. **18**: 299–321.

Gill, B.S., Friebe, B., and Endo, T.R. 1991. Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). Genome, **34**: 830–839.

Hsu, T.C. 1973. Longitudinal differentiation of chromosomes. Annu. Rev. Genet. **7**: 153–176.

Hutchinson, J., and Seal, A.G. 1983. A sequential *in situ* hybridization and C-banding technique. Heredity, **51**: 507–509.

Lawrence, J.B., Singer, R.H., and McNeil, J.A. 1990. Interphase and metaphase resolution of different distances within the human dystrophin gene. Science, (Washington, D.C.), **249**: 928–932.

Le, H.T., Armstrong, K.C., and Miki, B. 1989. Detection of rye DNA in wheat–rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Mol. Biol. Rep. **7**: 150–158.

Lichter, P., Boyle, A.L., Cremer, T., and Ward, D.C. 1991. Analysis of genes and chromosomes by nonisotopic *in situ* hybridization. Genet. Anal. Tech. Appl. **8**: 24–35.

McNeil, J.A., Johnson, C.V., Carter, K.C., Singer, R.H., and Lawrence, J.B. 1991. Localizing DNA and RNA within nuclei and chromosomes by fluorescence *in situ* hybridization. Genet. Anal. Tech. Appl. **8**: 41–58.

Mukai, Y., and Gill, B.S. 1991. Detection of barley chromatin added to wheat by genomic *in situ* hybridization. Genome, **34**: 448–452.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping of the 18S·26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.

Rayburn, A.L., and Gill, B.S. 1985. Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. J. Hered. **76**: 78–81.

Chromosoma (1996) 105:231–236

CHROMOSOMA

The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgene inserts in amphidiploid tobacco

E.A. Moscone*, M.A. Matzke, A.J.M. Matzke

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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Abstract. We have used combined fluorescent and genomic in situ hybridization (FISH/GISH) together with 4′,6-diamidino-2-phenylindole (DAPI) counterstaining to determine simultaneously the chromosome integration site and subgenomic allocation of a transgene in-sert in amphidiploid tobacco (*Nicotiana tabacum*, $2n = 4x = 48$). The procedure provides sufficient information on physical markers to identify at least 20 out of 24 chromosome pairs of two tobacco cultivars commonly used in studies on transgene expression and silencing (cv. Petit Havana SR1 and cv. Gatersleben). The chromosomes can be distinguished on the basis of diploid parental ancestry, size, morphology, the presence of rDNA loci and/or intergenomic exchanges, and the DAPI banding pattern, which is shown here for the first time for *N. tabacum*. From a single ISH experiment, it should now be possible in most cases to identify a tobacco chromosome carrying a transgene insert, thus permitting systematic studies of how the chromosome location of transgenes influences expression levels.

Introduction

transgene plants are used widely to study fundamental aspects of gene expression, and are becoming available commercially as the products of agricultural biotechnology. Although it is possible to obtain plant lines that stably express transgenes, there are numerous reported cases of unstable expression (Finnegan and McElroy 1994; Matzke and Matzke 1995) and progressive silencing of transgenes over generations (Kilby et al. 1992; Register et al. 1994). When examining factors that might influence transgene expression, an important consideration is the chromosome location. There are only a few cases, however, for which transgenes have been both localized to specific chromosome regions and analyzed in detail with respect to expression levels and inheritance patterns (Matzke et al. 1994; Park et al. 1996). Thus, even though significant progress has been made in refining in situ hybridization (ISH) techniques over the last ten years (Jiang and Gill 1994), molecular cytogenetic analyses have not yet been extensively exploited to study transgene germ plasm (Gill 1995).

Tobacco (*Nicotiana tabacum*) is used frequently for studies on transgene expression, primarily because it is relatively easy to transform with foreign genes and to regenerate entire fertile plants from single transformed cells. Transgene silencing has been studied intensively in tobacco at the genetic and molecular levels (Vaucheret 1993; Matzke et al. 1994; Park et al. 1996). In principle, therefore, transgene tobacco lines would provide abundant material for analyzing the relationship between the stability of gene expression and chromosome location. Progress in this endeavor, however, has been hindered by problems in distinguishing many chromosomes of tobacco, which is an amphidiploid ($2n = 4x = 48$) derived from two diploid progenitors [*N. sylvestris* (S genome) and either *N. tomentosiformis*, *N. otophora* or *N. tomentosa* (T genome)]. Recently, the use of genomic in situ hybridization (GISH) (Schwarzacher et al. 1989; Anamthawat-Jónsson and Reader 1995; Bennett 1995) has led to improvements in identification by permitting the categorization of tobacco chromosomes according to whether they belong to the S or T subgenome, and whether they contain rDNA loci or intergenomic exchanges (Kenton et al. 1993; Parokonny and Kenton 1995). Detecting single copy or low copy number transgene inserts by fluorescent in situ hybridization (FISH) can also be difficult, although transgene loci ranging in length from ca 10 kb (Wang et al. 1995) to 17 kb (Ambros et al. 1986a, b) have been localized on plant chromosomes. Recently, a 2.7 kb probe has been used to localize a transgene insert by FISH in *Petunia* (Fransz et al. 1996).

Despite the promise held by transgene tobacco plants for studying correlations between transgene expression and chromosome location, what has been lacking up until now is an uncomplicated ISH procedure that permits concomitant visualization of the transgene insert together with markers for chromosome identification. Here, we demonstrate that FISH and GISH can be combined to detect simultaneously the chromosome integration site and subgenomic allocation of a transgene locus in tobacco. (In this paper, we reserve the term FISH for detection of single transgene loci using plasmid probes, and GISH

* *Permanent address:* Instituto Multidisciplinario de Biología Vegetal (IMBIV), Casilla de Correo 495, 5000 Córdoba, Argentina

Edited by: D. Schweizer

Correspondence to: A.J.M. Matzke (e-mail: amatzke@oeaw.ac.at)

to refer to the use of genomic probes to paint separately the two tobacco subgenomes.) Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) reveals a distinctive heterochromatic banding pattern for most of the chromosomes. The cumulative information obtained from this procedure should allow in most cases the precise identification of tobacco chromosomes carrying transgene inserts.

Materials and methods

Plant material. Three transformed tobacco plants (*N. tabacum*) were used in this study. One plant was derived from the cultivar Gatersleben (H. Vaucheret, personal communication) and contained the transgene locus *271* in the homozygous condition. *271* is a multipurpose silencing locus that can inactivate any gene under the control of either the 35S promoter or the 19S promoter of cauliflower mosaic virus, as well as endogenous nitrite reductase genes of tobacco (Vaucheret 1993). It has been used extensively to study silencing mechanisms (Park et al. 1996). The two other plants were derived from the cultivar Petit Havana SR1 and corresponded to lines H9 and H59 (Neuhuber et al. 1994). These plants all had normal chromosome complements ($2n = 48$). The chromosome location of the transgene inserts in lines H9 and H59, which contain a different construct than line 271, will be reported separately.

Chromosome preparation from root tips. Tobacco plants, obtained from seeds or cuttings, were grown in hydroponic culture. Collected root tips (5–10 mm long) were pretreated with *p*-dichlorobenzene-saturated water in the dark for 2 h at room temperature (RT), and then fixed in freshly prepared 3+1 (3 parts 96% ethanol plus 1 part glacial acetic acid) for a minimum of 12 h at 4°C. For longer storage, root tips were kept at –20°C. After 1 month, 3+1 was replaced with 70% ethanol.

chromosome spreads for ISH were prepared as described by Park et al. (1996). Before squashing in 45% acetic acid, root apices were macerated in an enzyme solution (2% cellulase, 1% pectinase in 0.01 M citrate buffer, pH 4.8) for 20 min. After removal of the coverslip, slides were air-dried for 1–2 days at RT. Before hybridization, the spreads were pretreated with RNase A (Sigma, Munich) (100 µg/ml in 2×SSC) for 60 min at 37°C. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) The slides were then washed three times for 5 min with 2×SSC and digested with a freshly made proteinase K solution (0.1 µg/ml in 20 mM TRIS-HCl, pH 7.5, 2 mM $CaCl_2$) (Boehringer Mannheim, Vienna) at 37°C for 10 min (Fransz et al. 1996) followed by a rinse in 50 mM $MgCl_2$ in 1×PBS (10×PBS is 1.3 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4). Slides were fixed in 4% paraformaldehyde in 1×PBS for 10 min, then rinsed in 1×PBS and dehydrated in a 70%, 96% and 100% ethanol series (P. Fransz, personal communication).

Labeling of DNA probes, and conditions for ISH. DNA probes consisted either of the pRiN transgene construct, which is present in multiple copies at the *271* locus (Vaucheret et al. 1992) or total genomic DNA isolated from *N. sylvestris* or *N. otophora*. The plant genomic DNA had been sheared by 20 freeze/thaw cycles (Kenton et al. 1993). The DNA samples were labeled by nick translation, replacing dTTP with either biotin-11-dUTP (Sigma, Munich) or digoxigenin-11-dUTP (Boehringer Mannheim, Vienna). Labeled DNA probes were denatured at 95°C for 10 min. Hybridization mixes contained 60% formamide, 5% dextran sulfate, 2×SSC, 0.01% salmon sperm DNA and probes at a concentration of 0.4 or 0.8 ng/µl of hybridization solution for transgene construct pRiN or plant genomic DNA, respectively. Hybridization mixes were heated to 75°C for 10 min, then put on ice for 5 min.

Pretreated slides were denatured on a hot plate at 80°C for 10 min. Five microliters of hybridization mix was applied to a 22×22 mm coverslip, sealed with rubber solution, denatured at 80°C for 10 min, and incubated overnight at 37°C in a wet chamber.

Washing, blocking and fluorochrome detection. The slides were washed at 37°C in 60% formamide, 2×SSC for 15 min followed by a wash at 37°C in 2×SSC for 15 min. After a blocking step in 1×PBS, 3%BSA (bovine serum albumin) at 37°C for 30 min, biotin- and digoxigenin-labeled probes were detected as described by Strehl and Ambros (1993): in short, the first set of antibodies consisted of Dako-biotin (monoclonal mouse Dakopatts no. M743, Dako, Glostrup, Denmark) and sheep anti-digoxigenin-FITC (fluorescein isothiocyanate) (Boehringer Mannheim no. 1207741) in 1×PBS, 3% BSA. The second set of antibodies consisted of rabbit anti-mouse-TRITC (tetramethyl-rhodamine isothiocyanate) (Dakopatts no. R270) and rabbit anti-sheep-FITC (Dakopatts no. F135) in 1×PBS, 3%BSA. For both sets of antibodies, slides were incubated at 37°C for 45 min. Following each incubation step, slides were washed three times for 2 min each at RT with 1×PBS, 0.1% BSA.

The spreads were then counterstained/fixed for 3 min with DAPI staining solution, which contains 1 µg/ml DAPI in McIlvaine buffer (9 mM citric acid, 80 mM Na_2PO_4, 2.5 mM $MgCl_2$), 4% paraformaldehyde, 1×PBS. The slides were then rinsed with water, dehydrated through a 70%, 96%, and 100% ethanol series, air dried, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, California).

Fluorescence microscopy, and image acquisition. After ISH, chromosomes were viewed with a Leitz Laborlux-S fluorescence microscope equipped with a MonoCool View CCD camera (Photonic Science, Kent, England). Red, green, and blue images were captured in black and white using N2.1, I3, and A filters for TRITC, FITC, and DAPI, respectively. The images were combined and pseudocolored in the computer using Image Pro Plus Software provided with a Create RGB Macro. Photographs were printed with a Polaroid TX-2000 video printer. Black and white photographs of DAPI fluorescence were taken with Leitz-Vario Orthomat2 Camera System, using Kodak T-MAX 400 ASA film.

Karyotypic analysis. Five metaphase plates per cultivar were used for chromosome measurements after double GISH plus DAPI counterstaining. In the idiograms, the chromosomes were arranged according to the system proposed by Parokonny and Kenton (1995), although the number assigned here to each chromosome does not always imply homology with the one assigned by these authors.

Results

Localization of the transgene insert

Simultaneous FISH and GISH to somatic metaphase chromosomes of a transformed tobacco plant (*N. tabacum* cv. Gatersleben) were performed to determine, respectively, the chromosome integration site of the *271* transgene locus and its subgenomic allocation. A biotin-labeled pRiN probe containing sequences specific for the transgene locus was detected with rhodamine (red)-labeled antibodies; digoxigenin-labeled total genomic DNA probes of *N. sylvestris* (Fig. 1B) or *N. otophora* (Fig. 1D), which hybridize to the S or T fraction of the tobacco genome, respectively, were detected with fluorescein (green)-labeled antibodies. The chromosomes were counterstained with DAPI (Fig. 1A, C).

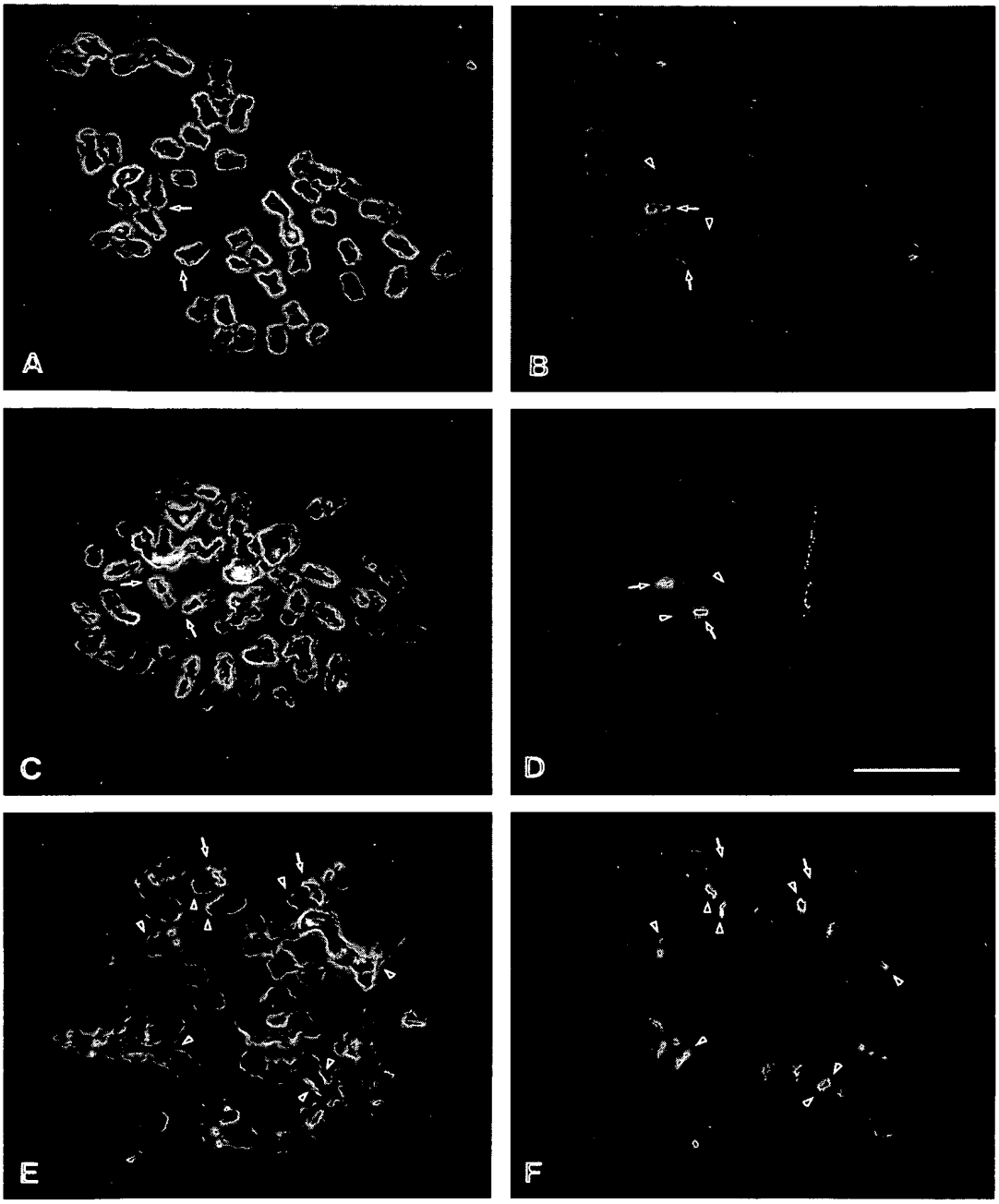

Fig. 1. **A–D** Fluorescence in situ hybridization/genomic in situ hybridization (FISH/GISH) to somatic metaphases of *Nicotiana tabacum* cv. Gatersleben ($2n = 48$). *Orange-red* (**B**) or *yellow* (**D**) fluorescent spots indicate hybridization to the biotin-labeled pRiN probe specific for the transgene insert, and *green* fluorescence indicates hybridization to the digoxigenin-labeled genomic DNA probes of *N. sylvestris* (S subgenome) (**B**) or *N. otophora* (T subgenome) (**D**). Unlabeled chromatin, which does not fluoresce, appears brown. **A, C** 4',6-Diamidino-2-phenylindole (DAPI) counterstaining of the same metaphases as in **B** and **D**, respectively. In **A** and **B**, three chromosomes are missing. Hybridization signals corresponding to the pRiN probe in **D** are yellow, not red, owing to green fluorescent painting of the chromosomes carrying the transgene insert. *Arrows* point out the location of the transgene insert. *Arrowheads* in **B** and **D** show hybridization of both genomic probes to the T3 nucleolus organizing regions (NOR)s. **1 E–F** Dual GISH to somatic metaphases of *N. tabacum* cv. Gatersleben ($2n = 48$). In **F**, *red* fluorescence shows hybridization to the biotin-labeled *N. sylvestris* total DNA probe (S subgenome); *green* fluorescence shows hybridization to the dioxigenin-labeled *N. otophora* total DNA probe (T subgenome). rDNA sites appear *yellow* owing to hybridization to both genomic probes. **E** DAPI counterstaining of the same metaphase as in **F**. *Arrows* indicate the NOR-bearing T3 pair; *arrowheads* show the eight rDNA loci (two of these are head-to-head; lower right). Any additional yellow spots are due to overlapping red and green painted chromosomes. Bar represents 10 μm

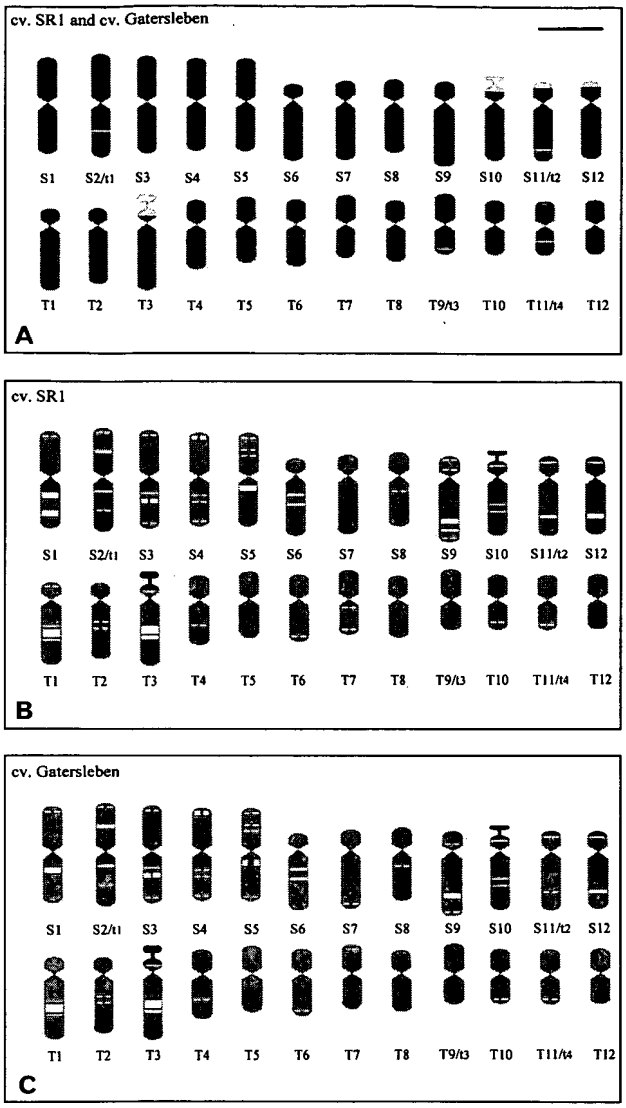

Fig. 2A–C. Karyotypic diagrams of *N. tabacum* cv. Petit Havana SR1 and cv. Gatersleben showing genomic painting (**A**) and DAPI banding patterns (**B, C**). **A** *Red* and *green* regions indicate S and T subgenomes, respectively; *yellow* regions show rDNA sites. **B, C** *White* and *black* segments indicate DAPI bright and dull heterochromatic bands, respectively; *blue areas* show euchromatic regions of indifferent fluorescence. Bar represents 3 μm

The FISH signal of the *271* transgene locus was observed on the long arm telomeres of a nucleolus organizing region (NOR)-bearing chromosome pair as reported previously (Park et al. 1996). Here, we have further refined the position of this transgene by localizing it to the T subgenome (Fig. 1A–D). This pair is one of the longest subtelocentrics of the tobacco karyotype (T3; Fig. 2). In addition to its active rDNA site, another marker is the presence of a large single (occasionally observed as a double) DAPI-positive heterochromatic band, which is in an intercalary position on the long arms (Figs. 1A, C, E, 2C).

Physical markers aiding in the identification of tobacco chromosomes

Dual GISH was applied to the 271 plant (cv. Gatersleben) (Fig. 1F), and to plants of cv. Petit Havana SR1. For this analysis, the probes consisted of *N. sylvestris* genomic DNA labeled with biotin and detected with rhodamine-labeled antibodies and *N. otophora* genomic DNA labeled with digoxigenin and detected with fluorescein-labeled antibodies. After GISH, the chromosomes were counterstained with DAPI (Fig. 1E). The idiograms derived from the karyotypic analysis are shown in Fig. 2. Both cultivars displayed the same karyotype after double GISH, which distinguished two subsets of differentially painted chromosomes corresponding to the S and T subgenomes of tobacco. Twelve pairs fluoresced predominantly or completely red because they hybridized to the biotin-labeled total genomic DNA from *N. sylvestris* (S subgenome). The remaining 12 pairs fluoresced mostly or completely green after hybridization to digoxigenin-labeled total genomic DNA from *N. otophora* (T subgenome). Four chromosome pairs were found to be homozygous for intergenomic translocations: S2/t1, S11/t2, T9/t3 and T11/t4. rDNA loci are present on the short arms of four chromosome pairs: three of them are from the S subgenome, S10, S11/t2, and S12, and one pair, T3, is from the T subgenome. These rDNA sites appear yellow after dual GISH because they hybridize to both genomic probes. The rDNA sites will also fluoresce when only one genomic probe is used – regardless of whether this probe is derived from *N. sylvestris* or *N. otophora* – because the rDNA loci from the two subgenomes are homologous (Fig. 1B, D). In these cases, the color of the rDNA loci depends on the fluorochrome used to detect the genomic DNA probe. The rDNA region located on T3 of both the Gatersleben and SR1 cultivars, and that on S10 of SR1 are associated with secondary constrictions, and can thus be considered NORs.

In cv. Petit Havana SR1 the DAPI banding pattern is most complex in the large metacentrics of the S subgenome S1–S5, where one or more intercalary bands of different size as well as terminal bands are present in one or both arms.The differences in the DAPI banding pattern allow the large metacentrics of the S subgenome, which otherwise lack markers (with the exception of the translocated T chromatin on S2), to be distinguished from each other.

Most of the subtelocentrics *S6, S7, S9 to S12; T1 to T3* display one or more intercalary DAPI-positive segments in the long arm; band complexes in S9, T1, and T3 are the largest of the karyotype. Terminal bands corresponding to the rDNA loci are DAPI-dull.

The small chromosomes [*small submetacentrics (S8, T4, T6, and T8) and metacentrics (T5, T7, and T9/t3 to T12*] – all of which, with the exception of S8, belong to the T subgenome – are relatively deficient in DAPI

bands. Small intercalary bands are located on the long arms of submetacentrics S8 and T4 and metacentrics T7 and T10. Tiny DAPI-enhanced dots were found at the long arm telomeres of the submetacentric T6 and the metacentrics T7 and T11/t4.

Five chromosome pairs of cv. Petit Havana SR1 are unbanded after DAPI: S7, T5, T8, T9/t3, and T12. However, because S7 alone belongs to the S subgenome and T9/t3 is a recombinant chromosome, difficulties in identification remain only for T5, T8, and T12.

Differences in the DAPI banding pattern of cv. Gatersleben in comparison with cv. Petit Havana SR1 were found for the following chromosomes: S1, S5, S7, S9, S11/t2 , S12, T1, T7 and T10. In this Gatersleben sample, five chromosomes lack DAPI banding: T5, T7, T8, T9/t3, and T12.

Discussion

To take full advantage of tobacco for investigating the relationship between transgene expression and chromosome location, reliable chromosome identification is required in addition to streamlined ISH procedures. However, the use of molecular cytogenetic techniques to analyze transgene tobacco lines has been hampered by difficulties in identifying many tobacco chromosomes and by time-consuming sequential FISH/GISH procedures. Here, we show that these problems can be largely overcome by performing combined FISH/GISH along with DAPI counterstaining. With the information on physical markers available from this technique, it is possible to distinguish 20–21 out of 24 chromosome pairs of tobacco. The significantly improved amenability of transgene tobacco to molecular cytogenetic analysis will allow researchers to address fundamental questions such as whether transgenes insert into heterochromatic regions (e.g. DAPI bands) and remain active there, and whether the S and T subgenomes are equally frequent targets for transgene integration. Moreover, the localization of transgene inserts on different tobacco chromosomes will provide molecular markers that are useful for gene mapping.

The GISH and DAPI karyotypes for two tobacco cultivars that have been used extensively for studies on transgene expression, cvs. Petit Havana SR1 and Gatersleben, are presented here. For SR1, only three small chromosomes of the T subgenome (T5, T8, and T12) are deficient in markers and difficult to distinguish. Even in these cases, however, slight differences in size and morphology – T5 is the largest and T12 the smallest in the group of small metacentrics, while T8 is submetacentric – can be of value in identification efforts. For Gatersleben, four small chromosomes remain indistinguishable: the three mentioned above for SR1 and in addition, T7, which lacks the two DAPI bands that are visible in SR1.

The combined FISH/GISH procedure was used to detect simultaneously the chromosome integration site and subgenomic allocation of the transgene locus, *271*. This locus has previously been localized on the long arm telomere of an NOR-bearing chromosome (Park et al. 1996). GISH has now allowed us to identify this chromosome as T3 according to the tobacco karyotype presented here and that described for another cultivar, 095–55 (Parokonny and Kenton 1995). Although the *271* locus is estimated to comprise 6–7 copies of the 8.3 kb pRiN transgene construct (Park et al. 1996), which would add up to ca 50–60 kb, preliminary data indicate that combined FISH/GISH can be used to localize tobacco transgene inserts that contain 1–2 copies of a construct that is ca 10 kb (E.A. Moscone and A.J.M. Matzke, unpublished results).

Together with a ca 30 kb transgene locus that has recently been detected in the S subgenome in an aneuploid tobacco line (Papp et al. 1996), the *271* locus is among the first to be localized to one of the subgenomes of tobacco. From these two examples, it appears that each subgenome can be a target for the integration of transgenes. Recently, a 20–25 kb gemini virus-related DNA sequence (GRD) has been localized on a small chromosome pair identified as T4 from the T subgenome of tobacco (cv. SR1) using sequential FISH/GISH (Kenton et al. 1995; Parokonny and Kenton 1995).

GISH distinguishes strikingly between the two subgenomes of tobacco. Because in single-color GISH experiments, all four rDNA loci will hybridize to either an *N. sylvestris* or an *N. otophora* probe, it is necessary to distinguish these signals from intergenomic exchanges. This is possible, though, because the rDNA sites are at the end of the short arms of subtelocentrics (S10, S11/t2, S12, and T3), whereas translocations are either at the end of the long arm of a large metacentric (S2/t1), a subtelocentric (S11/t2) or small metacentrics (T9/t3, T11/t4).

We observed four recombinant chromosome pairs in the SR1 and Gatersleben karyotypes. This is one fewer than reported previously for SR1 (Parokonny and Kenton 1995). The S2/t1 and S11/t2 translocated pairs in our karyotypes clearly correspond, respectively, to those denoted S2/ST1 and S11/ST2 by Parokonny and Kenton (1995). The relationship between our T9/t3 and T11/t4 recombinants to those described previously is less certain. They may correspond to T9/TS3 and T11/TS6, respectively, although according to Parokonny and Kenton (1995), the latter pair contains intergenomic exchanges in both arms, which we did not observe in T11/t4. Kenton and coworkers also applied DAPI counterstaining after FISH in tobacco (Kenton et al. 1993), but unlike the complete karyotypes shown here, they presented the banding pattern only for three rDNA-bearing pairs, ST2, S2, and T1 (these correspond, respectively, to the pairs we have designated S11/t2, S12, and T3).

In summary, considerable information on the chromosome location of transgene inserts in tobacco can be obtained in a single ISH experiment together with DAPI counterstaining: (1) FISH reveals the chromosome integration site of the transgene; (2) GISH indicates whether the chromosome containing the transgene is specific to the S or T subgenome and allows the detection of intergenomic translocation chromosomes; (3) DAPI counterstaining reveals a complex pattern of heterochromatic regions that serve as chromosome land-

marks. With this approach, tobacco thus becomes a tractable model plant to use in molecular cytogenetic analyses of transgene germ plasm.

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Reference

Ambros PF, Matzke AJM, Matzke MA (1986a) Localization of *Agrobacterium rhizogenes* T-DNA in plant chromosomes by *in situ* hybridization. EMBO J 5: 2073–2077

Ambros PF, Matzke MA, Matzke AJM (1986b) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 94: 11–18

Anamthawat-Jónsson K, Reader SM (1995) Pre-annealing of total genomic DNA probes for simultaneous genomic in situ hybridization. Genome 38: 814–816

Bennett MD (1995) The development and use of genomic in situ hybridization (GISH) as a new tool in plant biosystematics. In: Brandham PE, Bennett MD (eds) Kew Chromosome Conference IV. Royal Botanic Gardens, Kew, England, pp 167–183

Finnegan J, McElroy D (1994) Transgene inactivation: plants fight back! Biotechnology 12: 883–888

Fransz PF, Stam M, Montijn B, Ten Hoopen R, Wiegant J, Kooter JM, Oud O, Nanninga N (1996) Detection of single-copy genes and chromosome rearrangements in *Petunia hybrida* by fluorescence in situ hybridization. Plant J 9: 767–774

Gill BS (1995) The molecular cytogenetic analysis of economically important traits in plants. In: Brandham PE, Bennett MD (eds) Kew Chromosome Conference IV. Royal Botanic Gardens, Kew, England, pp 47–53

Jiang J, Gill BS (1994) Nonisotopic in situ hybridization and plant genome mapping: the first ten years. Genome 37: 717–725

Kenton A, Parokonny AS, Gleba YY, Bennett MD (1993) Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. Mol Gen Genet 240:159–169

Kenton A, Khashoggi A, Parokonny A, Bennett MD, Lichtenstein C (1995) chromosome location of endogenous geminivirus-related DNA sequences in *Nicotiana tabacum* L. Chromosome Res 3: 346–350

Kilby NJ, Leyser HM, Furner IJ (1992) Promoter methylation and progressive transgene inactivation in *Arabidopsis*. Plant Mol Biol 20: 103–112

Matzke AJM, Neuhuber F, Park Y-D, Ambros PF, Matzke MA (1994) Homology-dependent gene silencing in transgene plants: epistatic silencing loci contain multiple copies of methylated transgenes. Mol Gen Genet 244: 219–229

Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous (trans)genes? Plant Physiol 107: 679–685

Neuhuber F, Park Y-D, Matzke AJM, Matzke MA (1994) Susceptibility of transgene loci to homology-dependent gene silencing. Mol Gen Genet 244: 230–241

Papp I, Iglesias VA, Moscone EA, Michalowski S, Spiker S, Park Y-D, Matzke MA, Matzke AJM (1996) Structural instability of a transgene locus in tobacco is associated with aneuploidy. Plant J (in press)

Park Y-D, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM, Matzke MA (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J 9: 183–194

Parokonny AS, Kenton AY (1995) Comparative physical mapping and evolution of the *Nicotiana tabacum* L. karyotype. In: Brandham PE, Bennett MD (eds) Kew Chromosome Conference IV. Royal Botanic Gardens, Kew, England, pp 301–320

Register JC, Peterson DJ, Bell PJ, Bullock WP, Evans IJ, Frame B, Greenland AJ, Higgs NS, Jepson I, Jiao S, Lewnau CJ, Sillick JM, Wilson HM (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol Biol 25: 951–961

Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) In situ localization of parental genomes in a wide hybrid. Ann Bot 64: 315–324

Strehl S, Ambros PF (1993) Fluorescence in situ hybridization with immunohistochemistry for highly sensitive detection of chromosome 1 aberrations in neuroblastoma. Cytogenet Cell Genet 63: 24–28

Vaucheret H (1993) Identification of a general silencer for 19S and 35S promoters in a transgene tobacco plant: 90 bp of homology in the promoter sequence are sufficient for trans-inactivation. C R Acad Sci Paris 316: 310–323

Vaucheret H, Kronenberger J, Lepingle A, Vilaine F, Boutin J-P, Caboche M (1992) Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. Plant J 2: 559–569

Wang J, Lewis ME, Whallon JH, Sink KC (1995) chromosome mapping of T-DNA inserts in transgene *Petunia* by in situ hybridization. transgene Res 4: 241–246

Journal of Biotechnology, 14 (1990) 333–344
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The pFF plasmids: cassettes utilising CaMV sequences for expression of foreign genes in plants

Marja C.P. Timmermans, Pal Maliga, Jeffrey Vieira * and Joachim Messing
Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA

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Summary

A plant expression cassette was constructed using the cauliflower mosaic virus 35S 5′ regulatory region with the enhancer duplicated and the 35S polyadenylation signal. Insertion of a polylinker between the transcription initiation and polyadenylation sites allows for easy cloning of genes. To test the usefulness of the cassette chimeric bacterial genes were prepared. The constructs were introduced into *Nicotiana tabacum* suspension culture cells by the particle bombardment process. Expression of the β-glucuronidase reporter gene was verified by histochemical staining. Stable kanamycin and hygromycin resistant transgenic lines were obtained after introduction of chimeric genes encoding the enzymes neomycin phosphotransferase and hygromycin B phosphotransferase, respectively. The number of stable transformants was approximately 2% of the cells that transiently expressed the β-glucuronidase reporter gene.

Plasmid, pFF; CaMV sequence; Foreign gene; Plant

Introduction

Expression cassettes are modules of 5′ and 3′ regulatory regions for the construction of gene fusions with prokaryotic or eukaryotic genes. They are used to analyze

Correspondence to: J. Messing, Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, NJ 08855-0759, U.S.A.
* *Present address*: Department of Medical Microbiology, Stanford University, School of Medicine, Stanford, CA 94305, U.S.A.

specific regulatory sequences, to serve as internal controls (Kuhlemeier et al., 1987) or to express marker genes for the selection of drug-resistant cell lines. Chimeric gene constructions utilized regulatory sequences obtained from T-DNA opine synthase genes (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983), from a variety of plant genes such as the small subunit of ribulose 1,5-biphosphate carboxylase (Herrera-Estrella et al., 1984; Broglie et al., 1984), or from cauliflower mosaic virus (CaMV). Because a high level of expression is often a critical factor, the relative strengths of some of these plant promoters have been evaluated. Quantitative measurements of transcript levels in transformed tobacco cells (Morelli et al., 1985; Harpster et al., 1988) or transgenic petunia plants (Sanders et al., 1987) showed that the CaMV 35S promoter is at least 30 times stronger than the widely used nopaline synthase (NOS) promoter.

The 35S promoter is a viral promoter isolated from cauliflower mosaic virus, a DNA virus that infects members of the *Cruciferae*. Information on the complete nucleotide sequence of CaMV (Gardner et al., 1981) and on the 35S transcriptional start site (Covey et al., 1981; Guilley et al., 1982) is available. Comparison of transcript levels of various deletion mutants of the 35S promoter identified the −343 to −46 upstream region as being responsible for the majority of the 35S promoter strength (Odell et al., 1985). Multiple *cis* regulatory elements were identified within this region not only regulating the level of transcription but also conferring tissue specificity and developmental control (Odell et al., 1987; Ow et al., 1987; Benfey et al., 1989; Fang et al., 1989). The −343 to −90 fragment was shown to be of interest because of its enhancer-like properties. When cloned 5′ of the NOS promoter, a 3-fold increase in transcription levels was measured (Odell et al., 1988). Duplication of the −343 to −90 sequences resulted in a 10-fold transcriptional enhancement (Kay et al., 1987).

Although construction of expression cassettes utilizing the 35S promoter has been reported before (Pietrzak et al., 1986; Topfer et al., 1987), these do not use the full potential of the 35S enhancer sequences. We have constructed cassettes consisting of the duplicated 35S enhancer, the promoter region and polyadenylation signal. The unique restriction sites outside the cassettes facilitate subcloning it into other vectors and make the 5′ and 3′ regulatory regions interchangeable with 5′ and 3′ sequences of other genes. A polylinker between the 5′ and 3′ *cis*-acting elements facilitates easy construction of chimeric genes.

The new expression cassette was tested by inserting the well characterized genes for the enzymes neomycin phosphotransferase type II (NPT II) (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983), hygromycin B phosphotransferase (HPH) (Van den Elzen et al., 1985; Waldron et al., 1985) and β-glucuronidase (GUS) (Jefferson et al., 1986). We employed the particle bombardment process to introduce these chimeric gene constructs into suspension culture cells of *Nicotiana tabacum*. Histochemical staining for GUS activity was used to monitor transient expression one day after bombardment. Gene fusions with the NPT II and HPH coding regions were tested by selection of kanamycin and hygromycin stable transgenic lines.

Materials and Methods

Bacterial strains and plasmids

DNA constructions were carried out in *E. coli* strain JV30 (Vieira and Messing, 1987). Single-stranded DNA template for mutagenesis was prepared in *E. coli* strain BW313 (Kunkel, 1985). Plasmid pUC120 is identical to plasmid pUC118 except that it has a *Nco*I site 5′ to the *Eco*RI site (Vieira, 1988).

DNA manipulations

All DNA manipulations were performed as described previously by Maniatis et al. (1982) except for the preparation of single-stranded DNA which was prepared according to Vieira and Messing (1987). Oligonucleotide directed in vitro mutagenesis was carried out as described by Zoller and Smith (1984) and Kunkel (1985).

Introduction of DNA into cells by the particle gun

Suspension cultures of *Nicotiana tabacum* (line XD) were maintained in 250-ml Erlenmeyer flasks containing 50 ml of RMS medium (Klein et al., 1988a) and agitated under continuous light at 28°C. The cells were subcultured approximately every 4 days. Suspension culture cells (5 ml) were evenly distributed over a Whatman No. 4 filter paper (diameter 5.5 cm) using a Buchner funnel. The filter paper with cells was then placed on a RMS plate (0.7% agarose).

Tungsten particles (1 μm; 25 mg) were cleaned in 1 ml absolute ethanol by sonication at maximum power for 10 min. The particles were sedimented by centrifugation (2 min), washed twice with sterile distilled water, and resuspended in 0.5 ml of sterile distilled water. Tungsten was prepared for transformation by mixing 25 μl of the suspension with 2 μg DNA, dissolved in 5 μl $T_{10}E_1$-buffer (10 mM Tris, pH 8.0, 1 mM EDTA), 25 μl 1.0 M $CaCl_2$ and 10 μl 0.1 M spermidine. The particle/DNA mixture was incubated on ice for 2 min and centrifuged for 1 min in the Eppendorf centrifuge after which 30 μl of the supernatant was removed. The remaining solution was sonicated for 1 or 2 s. Two μl of the DNA-coated tungsten suspension was placed on the microprojectile for each bombardment which was performed as described by Klein et al. (1988b).

GUS assay

Cells were incubated on the filter paper on RMS medium for approximately 24 h at 28°C following bombardment. The filter paper was then transferred to a sterile Petri dish. GUS substrate mixture containing potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), sodium phosphate buffer (0.1 M, pH 7.0), Triton X-100 (0.06%, v/v) and the synthetic GUS substrate, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (0.3%, w/v) (Jefferson, 1987; Klein et al., 1988b), was pipetted dropwise onto the cells in order to cover all cells. After incubation overnight at 37°C, the cells expressing GUS were counted under a dissecting microscope.

Selection of kanamycin or hygromycin resistant calli

Following bombardment, the cells were incubated on the filter paper on RMS medium for 2 d at 28°C without drug selection. The filter paper was then placed in

a sterile flask with 30 ml RMS medium (0.5% agarose) containing 50 μg ml^{-1} kanamycin or 30 μg ml^{-1} hygromycin. The cells were resuspended from the filter and pipetted on three RMS plates (10 ml suspension per plate) containing the same antibiotic and at the same concentration as the top layer. Resistant calli were identified after 3–5 weeks of incubation at 28°C. Resistance of the selected calli was verified by their ability to repeatedly grow on the same selective medium.

Southern analysis of resistant calli

Genomic DNA was isolated from 10–15 g of calli. The tissue was ground in 20 ml extraction buffer (100 mM Tris, pH 8.5, 100 mM NaCl, 20 mM EDTA and 1% Sarcosyl) using a homogenizer. The tissue homogenate was extracted twice with phenol/chloroform and once with chloroform alone, then the DNA was alcohol-precipitated. The pellet was resuspended in $T_{10}E_1$-buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and the DNA further purified on CsCl density gradients (Maniatis et al., 1982). DNA (5 μg) was digested with the appropriate restriction enzymes, electrophoresed through 1% agarose, and transferred onto nitrocellulose filter. Hybridisation was carried out with the coding region of the appropriate genes using nick-translated ^{32}P-labeled DNA. Digestions, electrophoresis, DNA transfer, nick translation and Southern blot hybridisation were performed as described by Maniatis et al. (1982).

Results

Construction of expression cassette pFF19

The 5′ and 3′ termini of the 35S transcript have been mapped with S1 nuclease and showed an overlapping sequence of 200 nucleotides (Covey et al., 1981; Guilley et al., 1982). The 35S 5′ region and polyadenylation signal could therefore be isolated on a single small DNA fragment. These regulatory sequences from the CaMV strain CM1841 (Gardner et al., 1981) were cloned as a *Hin*cII (7016)-*Bgl*II (7674) fragment into the *Hin*cII-*Bam*HI sites of pUC120 forming the clone pFF10 (Fig. 1). With the lacZ and 35S promoters being in opposite orientations, read through from the lacZ promoter was eliminated. To allow for easy cloning of the different genes in between the 35S 5′ and 3′ regions, it was necessary to introduce a multiple cloning site at that position. Oligonucleotide directed in vitro mutagenesis was used to introduce a unique *Xho*I site to separate the two regulatory regions. Subsequently, the remaining pUC120 polylinker sites were eliminated as described in the legend of Fig. 1, to enable the insertion of a unique polylinker in the *Xho*I site (see below). Duplication of the 35S enhancer sequence in pFF15 was achieved by cloning the 329 bp *Eco*RV-*Hin*dIII fragment from pFF14 into the *Hin*cII-*Hin*dIII sites from pFF12 (Fig. 1).

As a final step a multiple cloning site was introduced resulting in the vector pFF19 (Fig. 2). The polylinker of vector pFF19 derives from plasmid pIC20h (Marsh et al., 1984). The *Eco*RI, *Eco*RV and *Cla*I restriction sites were deleted from the pIC20h polylinker by digesting pIC20h with *Sst*I and ligation of the

plasmid. The sites were eliminated because the same sites are present at other positions in pFF19. The remaining polylinker was excised as a *Hin*dIII-mung bean nuclease-treated fragment and ligated into the *Xho*I site from pFF15 which was filled in with Klenow fragment. Removing the 5′ protruding ends generated by the *Hin*dIII restriction endonuclease rather than filling them in had two effects. It created a unique *Nsi*I recognition site adjacent to the *Sph*I site and more importantly, it prevented the reformation of the *Hin*dIII sites when ligated into the *Xho*I site filled in with Klenow fragment. Both the 35S 5′ region and the poly(A) addition signal therefore have unique flanking restriction sites allowing them to be easily substituted by 5′ and 3′ regulatory sequences of other genes.

Chimeric gene constructions

The GUS coding region was cloned as a *Pst*I fragment from pRAJ260 (Jefferson et al., 1986) into the *Pst*I site from pFF19 to produce pFF19G (Fig. 2).

Before cloning the Tn5 NPT II gene-coding region into our expression vector, we deleted the upstream ATG which is located 16 bp 5′ of the real translation initiation site. Deletion of this ATG ensures for initiation at the right start codon and therefore allows a higher level of drug selection (Rogers et al., 1985). The NPT II gene was digested with *Bcl*I-*Aha*II and ligated into *Bgl*II-*Cla*I digested pIC20h vector (Marsh et al., 1984) resulting in the plasmid pIC20h5K′. The 5′ end of the NPT II coding region was cloned as a 250 bp *Mbo*I-*Taq*I fragment into the *Bam*HI-*Cla*I sites of pIC20h forming pIC250neo. Since the *Mbo*I site is located in between the two ATGs, this 250 bp fragment only contains the right translational

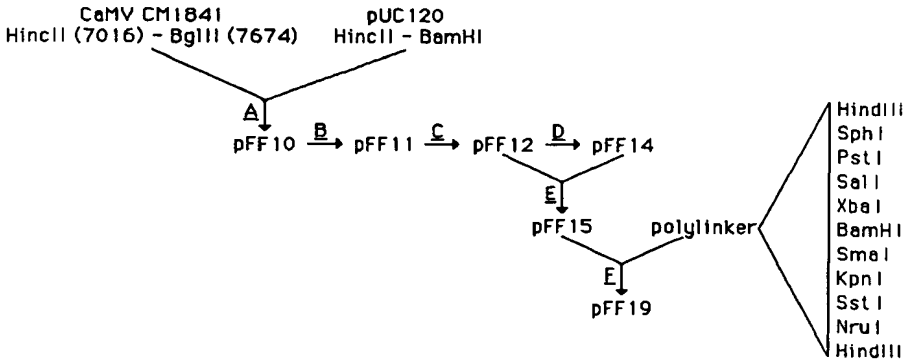

Fig. 1 Construction of plasmid pFF19. The 658 bp *Hin*cII-*Bgl*II fragment from the CaMV strain CM1841 was cloned into the *Hin*cII-*Bam*HI sites from pUC120 to yield pFF10 (A). Mutagenesis was used to change nucleotides 7477 and 7479 (CM1841 map positions) from Ts to Gs, creating a unique *Xho*I site (7474) yielding pFF11 (B). The flanking *Sst*I, *Kpn*I and *Sma*I restriction sites were deleted by digestion with *Sst*I and *Sma*I, treatment with the Klenow fragment and ligation of the blunt ends yielding pFF12 (C). Similarly, the *Hin*cII, *Pst*I and *Sph*I sites were deleted yielding pFF14 (D). The 35S enhancer was duplicated by inserting the *Eco*RV-*Hin*dIII fragment from pFF14 into the *Hin*cII-*Hin*dIII sites of pFF12 yielding pFF15 (E). The polylinker was inserted as a Mung Bean nuclease treated *Hin*dIII fragment into the *Xho*I site of pFF15 which was filled in with Klenow fragment yielding pFF19 (F).

start site. The unique *Pst*I site from the NPT II gene is also present on this fragment and was used to combine the 3′ end of the gene with this isolated 5′ end. To do so pIC20h5K′ was digested partially with *Pst*I and completely with *Xho*I. The 3.3 kb fragment was isolated and ligated to the 250 bp *Pst*I-*Sal*I fragment from pIC250neo. The resulting clone, pICNeo6, has the entire coding sequence from the Tn5 NPT II gene with the upstream ATG removed. It was digested with *Eco*RI, treated with Mung Bean nuclease and subsequently digested with *Sst*I. To construct the plasmid pFF19K, this fragment was cloned into the *Sst*I and *Sph*I (blunt-ended with Klenow fragment) sites of pFF19 (Fig. 2).

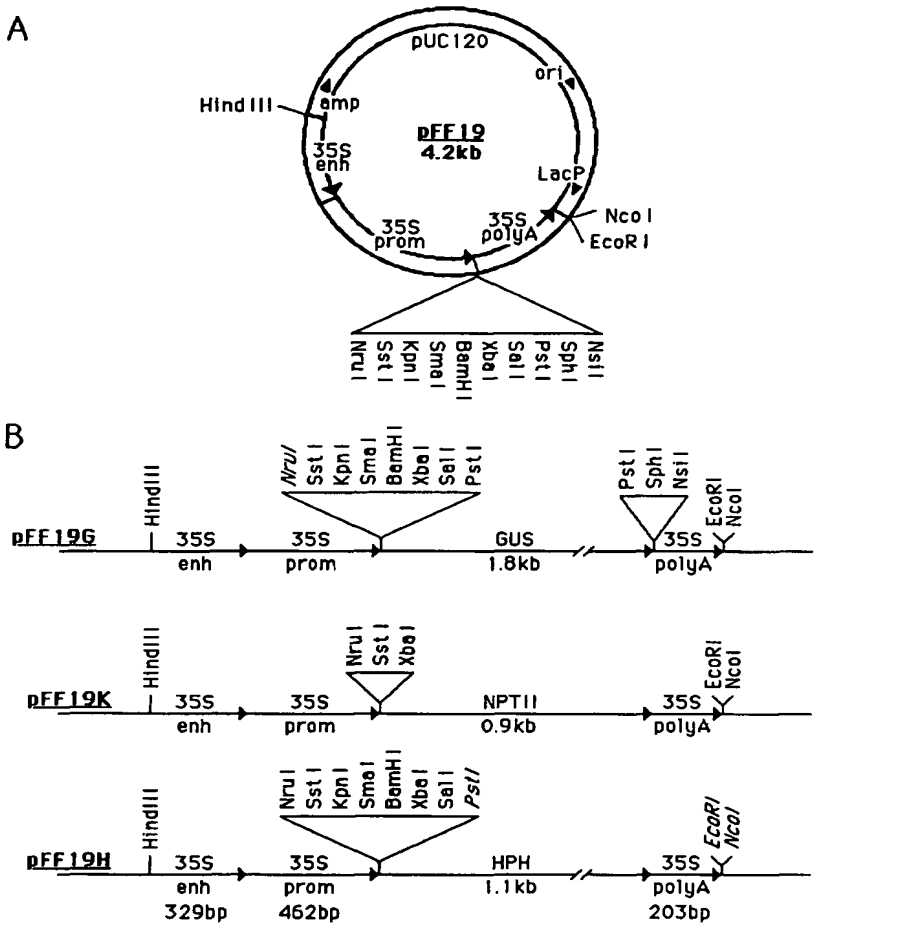

Fig. 2. The expression cassette pFF19 (A) and chimeric genes to express the GUS, NPT II and HPH enzymes (B). The direction of transcription of the regulatory and coding regions is indicated by the arrows. Restriction sites shown in italics are not unique because they are also present in the coding sequence of the reporter gene.

TABLE 1

The number of GUS-expressing cells and kanamycin or hygromycin resistant clones after bombardment with plasmids pFF19G, pFF19K and pFF19H, respectively

	pFF19G		pFF19K		pFF19H	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
	126	336	5	5	5	3
	153	268	2	3	2	4
	57	109	6	2	3	4
	138	418	1	5	2	3
			1	2	1	5
			2	3	2	7
			2	4	2	3
			1	3	4	4
			5	6	5	2
			-	5	3	3
Average	119	283	2.5	3.8	2.9	3.8

Plasmid pJD214Hy (Dougherty and Temin, 1986) was used to clone the HPH coding region into our expression cassette. This plasmid contains the HPH gene from pLG89 (Gritz and Davies, 1983) flanked at the 5′ end by a polylinker which includes a *Xba*I, *Sal*I and *Pst*I site and at the 3′ end by a *Cla*I site. The 1.1 kb *Cla*I-*Sal*I fragment from pJD214Hy was ligated into *Sph*I-*Sal*I digested pFF19 after treating the protruding ends of the *Cla*I and *Sph*I restriction sites with Klenow fragment (Fig. 2). In the resulting plasmid pFF19H, there are no unique sites flanking the 35S polyadenylation signal, because of the presence of both an *Eco*RI and a *Nco*I site within the hygromycin coding sequence.

Transient gene expression assay with pFF19G

The chimeric construct pFF19G was introduced into *N. tabacum* suspension culture cells by bombardment with the particle gun to analyze if the 35S regulatory regions of the pFF19 expression cassette would lead to transient expression of the chimeric gene. After a 1-d incubation, the bombarded cells were treated with the GUS histochemical substrate mixture. GUS expressing cells develop a distinctive blue color which can be easily detected microscopically. The number of blue cells in independent bombardments was determined and is given in Table 1. In two different experiments, an average of 119 (Experiment I) and 283 (Experiment II) cells expressed GUS, with a variation between 57 and 418 blue cells per bombardment.

Selection of kanamycin and hygromycin resistant clones

In order to test the efficiency of the chimeric NPT II and HPH genes for obtaining stable transformants, XD cells were bombarded with plasmids pFF19K and pFF19H. The bombarded cells were incubated for 2 d without drug selection to allow expression of the resistance gene. Stable transformants were then selected by

embedding the cells in medium containing 50 μg ml^{-1} kanamycin or 30 μg ml^{-1} hygromycin, respectively. The number of kanamycin and hygromycin resistant colonies that was obtained per bombardment is listed in Table 1. On average, about three resistant clones were recovered in each bombarded sample.

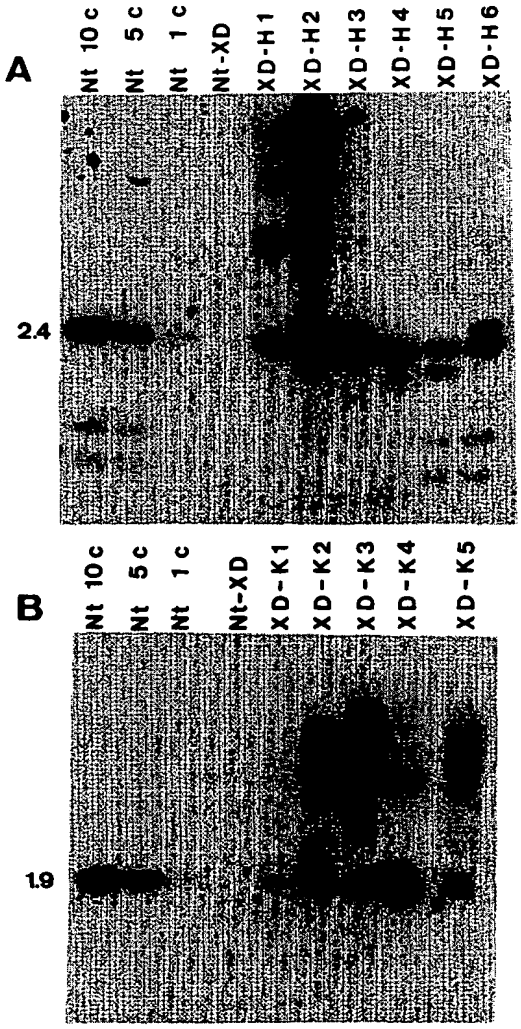

Fig. 3. Southern analysis of hygromycin (A) and kanamycin (B) resistant calli from transformed *N. tabacum* XD cells. Lane Nt-XD, DNA from nontransformed suspension culture cells. Lanes XD-H1 to XD-H6, DNA from hygromycin resistant call. Lanes XD-K1 to XD-K5, DNA from kanamycin resistant call. Lanes Nt 10c, Nt 5c and Nt 1c, genomic DNA mixed with pFF19H (A) or pFF19K (B) to reconstruct the integration of 10, 5 or 1 copy of the chimeric HPH (A) or NPT II (B) genes per diploid nucleus.

Southern analysis of stable transformants

DNA isolated from kanamycin and hygromycin resistant calli were probed for the presence of transforming DNA by the Southern procedure. DNA isolated from kanamycin resistant clones was digested with *Eco*RI-*Hin*dIII, and after transferring onto nitrocellulose filter probed with nick-translated *Eco*RI-*Hin*dIII insert from pFF19K. These two enzymes have unique recognition sites just outside the 35S regulatory regions producing a 1.9 kb fragment (Fig. 2). Whereas DNA from nontransformed tissue lacked hybridisation using this probe, all the kanamycin resistant calli showed a hybridizing band of the predicted size (Fig. 3). Comparison of the intensity of this band with reconstructed mix indicates that the transgenic clones carry 1 to 20 copies of the intact NPT II gene per diploid genome. DNA from hygromycin resistant colonies was analyzed in a similar manner. There are two *Pvu*II sites in the lacZ region, therefore digestion with the *Pvu*II restriction endonuclease excised the chimeric HPH gene from plasmid pFF19H as a 2.4 kb fragment. Nick-translated *Pvu*II insert from pFF19H was used as a probe resulting in a hybridizing band of 2.4 kb only in the lanes containing DNA from hygromycin resistant calli (Fig. 3). The number of intact HPH gene copies per diploid genome is around 3 to 30. Southern probing for both types of transgenic calli also revealed fragments with sizes deviating from the expected 1.9 or 2.4 kb. These are due to rearrangements of the plasmid DNA upon transformation, a phenomenon which has been observed before (Paszkowski et al., 1984; Klein et al., 1988a). The additional two bands seen in the first two lanes of Fig. 3A correspond to a size of approximately 1.0 and 1.4 kb and are most probably due to star activity of the restriction enzyme used.

Discussion

We described the construction of a new vector pFF19 utilizing the 35S promoter region and a duplicated enhancer as 5′ region to allow for high levels of transcription (Kay et al., 1987). The usefulness of the cassette was tested in a transient assay with the GUS reporter gene after introducing the plasmid pFF19G into tobacco suspension culture cells. Also stable transgenic lines were obtained using chimeric constructs carrying the selectable markers NPT II or HPH. Previous studies suggested that about 2–5% of the cells that transiently express a foreign gene stably integrate it (Klein et al., 1988a). Comparison of the number of GUS positive cells, as a measure of the success of DNA delivery, and of the number of kanamycin or hygromycin resistant clones, as the number of stable transformants, yielded a similar value. Efficiency of bombardment is variable (Table 1) and depends on a number of parameters (Klein et al., 1988b). Determining the number of GUS expressing cells is useful to compare efficiency of DNA delivery in independent experiments. In addition to the experiments described in this manuscript we tested expression of the GUS gene in pFF19G in suspension culture cells of *Avena sativa, Oryza sativa* and *Zea mays*. The number of GUS expressing cells was comparable to those obtained in *Nicotiana tabacum* (data not shown). This observation confirms

earlier findings which showed that the 35S upstream region is active both in transgenic tobacco and petunia plants (Odell et al., 1985; Jefferson et al., 1987; Sanders et al., 1987) as well as in a transient protoplast assay of several dicots and monocots (Fromm et al., 1985; On-Lee et al., 1986; Nagata et al., 1987).

The pFF19 cassette was designed to allow for maximum expression of a reporter gene based on the use of an efficient 5′ regulatory region. If desired, expression of genes in the cassette can be further improved. Alterations that may increase expression include exchange of sequences at the 3′ terminus (Ingelbrecht et al., 1989), insertion of an intron in between the transcription and translation initiation sites (Callis et al., 1987) and insertion of specific 5′ untranslated leader sequences at the same position (Gallie et al., 1987; Sleat et al., 1987; Jobling and Gehrke, 1987; Harpster et al., 1988). These sequences can be easily incorporated into our cassettes using the unique restriction sites present.

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References

Benfey, P.N., Ren, L. and Chua, N.-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. EMBO J. 8, 2195–2202.

Bevan, M.W., Flavell, R.B. and Chilton, M.-D. (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

Broglie, R., Coruzzi, G., Fraley, R.T., Rogers, S.G., Horsch, R.B., Niedermeyer, J.G., Fink, C.L., Flick, J.S. and Chua, N.-H. (1984) Light-regulated expression of a pea ribulose-1,5-biphosphate carboxylase small subunit gene in transformed plant cells. Science 224, 838–843.

Callis, J., Fromm, M. and Walbot, V. (1987) Introns increase gene expression in cultured maize cells. Genes Dev. 1, 1183–1200.

Covey, S.-N., Lomonossoff, G.P. and Hull, R. (1981) Characterization of cauliflower mosaic virus DNA sequences which encode major polyadenylated transcripts. Nucleic Acids Res. 9, 6735–6747.

Dougherty, J.P. and Temin, H.M. (1986) High mutation rate of spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. 168, 4387–4395.

Fang, R., Nagy, F., Sivasubramaniam, S. and Chua, N.-H. (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1, 141–150.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C. (1983) Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80, 4803–4807.

Fromm, M., Taylor, L.P. and Walbot, V. (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. USA 82, 5824–5828.

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. Nucleic Acids Res. 15, 3257–3273.

Gardner, R.C., Howarth, A.J., Hahn, P., Brown-Luedi, M., Shepherd, R.J. and Messing, J. (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Res. 9, 2871–2888.

Gritz, L. and Davies, J. (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25, 179–188.

Guilley, H., Dudley, R.K., Jonand, G., Balazs, E. and Richards, K.E. (1982) Transcription of cauliflower mosaic virus DNA detection of promoter sequences and characterisation of transcripts. Cell 30, 763–773.

Harpster, M.H., Townsend, J.A., Jones, J.D.G., Bedbrook, J. and Dunsmuir, P. (1988) Relative strengths of the 35S cauliflower mosaic virus, 1′, 2′ and nopaline synthase promoters in transformed tobacco, sugarbeet and oilseed rape callus tissue. Mol. Gen. Genet. 212, 182–190.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.-P., Van Montagu, M.C. and Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2, 987–995.

Herrera-Estrella, L., Van den Broeck, G., Maenhaut, R., Van Montagu, M.C., Schell, J., Timko, M. and Cashmore, A. (1984) Light-inducible and chloroplast-associated expression of a chimeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. Nature 310, 115–120.

Ingelbrecht, I.L.W., Herman, L.M.F., Dekeyser, R.A., Van Montagu, M.C. and Depicker, A.G. (1989) Different 3′ end regions strongly influence the level of gene expression in plant cells. The Plant Cell 1, 671–680.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986) β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci. USA 83, 8447–8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5, 387–405.

Jobling, S.A. and Gehrke, L. (1987) Enhanced translation of chimeric messenger RNAs containing a plant viral untranslated leader sequence. Nature 325, 622–625.

Kay, R., Chan, A., Daiy, M. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236, 1299–1302.

Klein, T.M., Harper, E.C., Svab, Z., Sanford, J.C., Fromm, M.E. and Maliga, P. (1988a) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc. Natl. Acad. Sci. USA 85, 8502–8505.

Klein, T.M., Gradziel, T., Fromm, M.E. and Sanford, J.C. (1988b) Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. Bio/Technology 6, 559–563.

Kuhlemeier, C., Green, P. and Chua, N.-H. (1987) Regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. 38, 221–257.

Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488–492.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32, 481–485.

Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose-1,5-biphosphate carboxylase small subunit of pea. Nature 315, 200–204.

Nagata, T., Okada, K., Kawazu, T. and Takebe, I. (1987) Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. Mol. Gen. Genet. 207, 242–244.

Odell, J.T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313, 810–812.

Odell, J.T., Nagy, F. and Chua, N.-H. (1987) Variability in 35S promoter expression between independent transformants. In: McIntosh, L. and Key, J. (Eds.), Plant Gene Systems and their Biology, Alan R. Liss, New York, 62, pp. 329–331.

Odell, J.T., Knowlton, S., Lin, W. and Mauvais, C.J. (1988) Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. Plant Mol. Biol. 10, 263–273.

On-Lee, T.M., Turgeon, R. and Wu, R. (1986) Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplast of rice, wheat and sorghum. Proc. Natl. Acad. Sci. USA 83, 6815–6819.

Ow, D.W., Jacobs, J.D. and Howell, S.H. (1987) Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by the firefly luciferase gene as a reporter of promoter activity. Proc. Natl. Acad. Sci. USA 84, 4870–4874.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B. and Potrykus, I. (1984) Direct gene transfer to plants. EMBO J. 3, 2717–2722.

Pietrzak, M., Shillito, R.D., Hohn, T. and Potrykus, I. (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res. 14, 5857–5868.

Rogers, S.G., Fraley, R.T., Horsch, R.B., Levine, A.D., Flick, J.S., Brand, L.A., Fink, C.L., Mozer, T., O'Connell, K. and Sanders, P.R. (1985) Evidence for ribosome scanning during translation initiation of mRNAs in transformed plant cells. Plant Mol. Biol. Rep. 3, 111–116.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Fraley, R.T. (1987) Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res. 15, 1543–1558.

Sleat, D.E., Gallie, D.R., Jefferson, R.A., Bevan, M.W., Turner, P.C. and Wilson, T.M.A. (1987) Characterisation of the 5′-leader sequence of tobacco mosaic virus RNA as a general enhancer of translation in vitro. Gene 60, 217–225.

Topfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H. (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res. 15, 5890.

Van den Elzen, P.J.M., Townsend, J., Lee, K.Y. and Bedbrook, J.R. (1985) A chimeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol. Biol. 5, 299–302.

Vieira, J. and Messing, J. (1987) Production of single-stranded plasmid DNA. Methods Enzymol. 153, 3–11.

Vieira, J. (1988) Ph.D. thesis, University of Minnesota U.S.A.

Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L. and Malcolm, S.K. (1985) Resistance to hygromycin B: a new marker for plant transformation. Plant Mol. Biol. 5, 103–108.

Zoller, M.J. and Smith, M. (1984) Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3, 479–488.

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CHROMOSOMA

Distribution of the rDNA and three classes of highly repetitive DNA in the chromatin of interphase nuclei of *Arabidopsis thaliana*

S. Bauwens[1], P. Van Oostveldt[2], G. Engler[1], and M. Van Montagu[1]

[1] Laboratorium voor Genetica, Faculteit van de Wetenschappen, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
[2] Laboratorium voor Biochemie, Faculteit van de Landbouwwetenschappen, Rijksuniversiteit Gent, Coupure Links 653, B-9000 Gent, Belgium

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Abstract. The distribution of the ribosomal RNA (rRNA) genes and three classes of highly repetitive DNA in the chromatin of interphase nuclei of *Arabidopsis thaliana* was studied for the first time through non-isotopic in situ hybridization and luminescence digital imaging microscopy. Each of the three classes of highly repetitive DNA exhibited a characteristic hybridization pattern, and one class was seen to be primarily localized on two chromocentres, which would allow it to distinguish a particular chromosome. The rDNA was consistently localized on the two largest chromocentres and on one or two smaller chromocentres. A limited number of nuclei exhibited more than four labelled chromocentres, indicative of either polypoidy or differential amplification of the rDNA. In nuclei where the nucleolus could be clearly observed, the nucleolar associated chromocentres (NACs) were seen to be labelled by the ribosomal DNA (rDNA) probe.

Introduction

It is clear from descriptive studies that the chromosomes are endowed with a higher order structure and that the organisation of chromosomes and chromatin within nuclei is not random (Hochstrasser et al. 1986; Hochstrasser and Sedat 1987a, b; Manuelidis and Borden 1988; Hiraoka et al. 1987, 1989; Oud et al. 1989; Van Dekken et al. 1989). Eukaryotic genomes are extremely complex and highly variable in DNA content and organisation (Gall 1981; Leutwiler et al. 1984; Gross and Garrard 1988; Meyne et al. 1990; Elgin 1990). The structure of chromosomes and chromatin has been studied with a variety of techniques. The development of recombinant DNA technology has made it possible to use in situ hybridization (ISH) techniques to visualize sequences as specific cytogenetic markers (Lichter and Ward 1990).

Offprint requests to: S. Bauwens

Among angiosperms, *Arabidopsis* has the smallest known genome (Meyerowitz and Pruitt 1985) and displays a characteristic chromatin distribution in the interphase nucleus. Most of the DNA is distributed in chromocentres the number of which corresponds roughly with the number of chromosomes (Bauwens and Van Oostveldt 1990). These chromocentres, which are present until early prophase, are also called prochromosomes. The chromocentres correspond to highly condensed heterochromatin which is generally thought to be present on either side of the centromere (Dyer 1979; Nagl 1979). The small genome size has made *Arabidopsis* attractive and successful as an object for gene analysis, but has made it less suitable for cytological analysis. However, the availability of very sensitive microscopical techniques such as non-isotopic ISH and the development of highly sensitive instruments such as low-light-level CCD cameras (CCD = charge coupled device) and confocal scanning laser microscopes (CSLMs) combined with powerful digital image analysis instruments, can drastically extend and enlarge the scope of our observations (Hiraoka et al. 1987; Agard et al. 1989; Brakenhoff et al. 1989; Arndt-Jovin and Jovin 1989).

The percentage of repetitive DNA in the nucleus of *Arabidopsis* is much smaller than in other plants. Moreover, *Arabidopsis* has much less interspersed repetitive DNA; the repetitive DNA sequences are mainly arranged in large tandem arrays and are thought to be present near the centromeres (Meyerowitz and Pruitt 1985; Simoens et al. 1988; Dyer 1979). The ribosomal RNA (rRNA) genes (Unfried et al. 1989; Unfried and Gruendler 1990) and the three classes of highly repetitive DNA identified by Martinez-Zapatar et al. (1986) and Simoens et al. (1988) represent the majority of the repetitive DNA in the nucleus of *Arabidopsis*. In this article we describe the physical distribution of the rRNA genes and the three classes of highly repetitive DNA sequences in *Arabidopsis* in the interphase nucleus using non-isotopic ISH and luminescence digital imaging microscopy.

Materials and methods

Preparation of the microscopical slides. Seeds from *Arabidopsis thaliana* (collection number C24; a generous gift from Dr. D. Van Der Straeten, Lab. Genetica, Fac. Wetenschappen, Rijksuniversiteit Gent, Belgium) were decontaminated (Valvekens et al. 1988) and germinated on Murashige & Skoog medium (mineral salts, no sucrose) at room temperature. Seedlings were harvested after 3 or 4 days, pretreated in 0.002 M 8-hydroxyquinoline for 30 min at room temperature and then another 30 min at 4° C, and fixed in 3:1 ethanol:acetic acid for 1 h at room temperature. After a time in 0.01 M citric acid/sodium citrate buffer, pH 4.8, the fixed seedlings were digested in 1% pectinase (Sigma, Mo., USA), 2% cellulase (Sigma), in the same buffer, for 30 min at 37° C. After removal of the enzyme solution the seedlings were rinsed in the citric acid buffer for 30 min at room temperature. They were then transfered to 10:1 ethanol:ether-cleaned slides, in 45% acetic acid. Most of the cotyledons were cut away and at least six root tips were put on each slide. The plant material was then squashed, the coverslip was removed on solid carbon dioxide, the preparations were allowed to air dry and were stored at −18° C until use (adapted protocol from Schwarzacher et al. 1980).

Labelling of the DNA probes. The three classes of highly repetitive DNA sequences from *Arabidopsis* are composed of tandemly repeated units of 180, 500 and 160 bp respectively, cloned into pGem-2 (Promega, USA), and were a generous gift of C. Simoens, Lab. Genetica, Fac. Wetenschappen, Rijksuniversiteit Gent, Belgium (Simoens et al. 1988). A representative of each of the three classes (the 180, 500 and 160 bp repeat respectively) was used in this experiment.

The ribosomal DNA (rDNA) from *Arabidopsis* used as a probe consisted of a mixture of four inserts, each cloned into pBS(I)KS$^+$ (Stratagene, USA) and comprising the 18 S, 5.8 S and 25 S rRNA genes, as well as the intergenic region (IGR), and was a generous gift from Dr. P. Gruendler, Institut für Botanik der Universität Wien, Austria (Unfried et al. 1989; Unfried and Gruendler 1990). Undigested constructions were labelled by nick translation with biotin-21-dUTP, according to the manufacturer's specifications (BRL Life Technologies, Md., USA).

In situ hybridization. The hybridization mixture contained 50% formamide, 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 300 ng/µl salmon sperm DNA, and biotinylated probe DNA (vector + insert) dissolved to a concentration of 2 ng/µl for the 180, 500 and 160 bp repeat sequences, and 8 ng/µl for the rDNA.

Slide preparations were digested with RNase (Sigma; 100 µg/ml, in 2 × SSC) for 1 h at 37° C. They were then washed (3 × 5 min in 2 × SSC), dehydrated (70%, 90%, 100% ethanol, 5 min, each) and dessicated (10 min). After this they were digested with 0.1 µg/ml proteinase K (Boehringer Mannheim, FRG) in proteinase K buffer (200 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4) for 10 min at 37° C. After a wash in proteinase K buffer (5 min) and a 5 min preincubation in 1 × PBS, 50 mM $MgCl_2$ [1 × PBS is 10 mM ($Na_2HPO_4 + NaH_2PO_4$), pH 7.0, 150 mM NaCl], they were post-fixed for 10 min in a freshly prepared solution of 4% (w/v) paraformaldehyde in 1 × PBS, 50 mM $MgCl_2$. After a wash in 1 × PBS (5 min) and a preincubation in 0.1 × SSC, they were denatured in 0.1 × SSC, at approximately 100° C for 5 min and then immediately transferred to ice cold absolute ethanol (10 min) and dessicated.

Probe DNA was denatured at approximately 100° C for 5 min, after which the Eppendorf tubes were immediately transferred to ice. Probe solution (10 µl) was applied to each slide under an 18 × 18 mm coverslip. Probe and target were denatured a further 5 min at 80°–85° C on a hot plate. The coverslip was not sealed. ISH took place at 37° C in a moist chamber, overnight (approximately 16 h). All washes were carried out at room temperature. (The ISH was based on the protocol described by Nederlof et al. 1989 b.)

Immunocytochemical detection. After hybridization, the slides were washed 3 × 5 min in 50% formamide, 2 × SSC, 2 × 5 min in 2 × SSC and 1 × 5 min in 4 × SSC, 0.05% Tween-20.

After this the slides were incubated for 10 min in 4 × SSC, 5% (w/v) non-fat dry milk (Gloria). This was followed by incubation with 5 µg/ml avidin D-FITC (fluorescein isothiocyanate; Vector Laboratories, Calif., USA) 4 × SSC, 5% non-fat dry milk (100 µl under a 24 × 60 mm coverslip) for 20 min, 3 × 5 min washing with 4 × SSC, 0.05% Tween-20, incubation with 5 µl/ml biotinylated goat-anti-avidin D (Vector Laboratories), 4 × SSC, 5% non-fat dry milk, (100 µl under coverslip) for 20 min, 3 × 5 min washing in 4 × SSC, 0.05% Tween-20 and a second incubation with avidin D-FITC as above. Slides were finally washed 2 × 5 min in 4 × SSC, 0.05% Tween-20, 1 × 5 min in 1 × PBS, dehydrated (70%, 90%, 100% ethanol), air dried and mounted in an anti-fade reagent [2% (w/v) DABCO (1,4-diazobicyclo-(2,2,2)-octane) in 90% glycerol 10% 200 mM Tris-HCl, 0.02% (w/v) NaN_3, pH 7.5] containing 0.5 µg/ml propidium iodide].

All washes and incubations were at room temperature. Incubations with avidin D-FITC and biotinylated goat-anti-avidin D were done in a moist chamber, in the dark.

Fluorescence microscopy. Images were recorded with the Bio-rad MRC-500 CSLM system. The CSLM from Bio-rad was fitted to an Orthoplan microscope from Leitz (FRG).

Excitation and dual wavelength detection were performed with the A_1/A_2 filter block combinations. The A_1 filter combination consists of a 514 DF 10 excitor filter for excitation with the 514 nm line of an Ar^+ ion laser (used at approximately 23% of maximum intensity) and a DR 527 LP dichroic reflector. The A_2 filter combination consists of a DR 565 LP dichroic reflector for the separation of the red propidium iodide, and the green FITC fluorescence light, and two barrier filters, an EF 600 LP filter ("red channel") and a 540 DF 30 filter ("green channel"). An Olympus 60×, NA 1.40, oil immersion lense (Olympus, Japan) was used.

Digital image processing was performed with the software provided with the Bio-rad MRC-500 CSLM system, and consisted of noise reduction filtering through averaging (KALMAN filtering) during image acquisition, and eventual contrast enhancement by subtraction of the mean of the background and scaling of the remaining image. Merging of the two images obtained by dual wavelength detection of the FITC and propidium iodide signals, was performed through the MERGE command.

Black and white photographs were taken from a high-resolution video display (7 in, flat screen, 16 MHz; Lucius & Baer, FRG) with a T-max 100 ASA film (Kodak, N.Y., USA; f=8; video display settings: brightness = 500, contrast = 100).

Results

Slide preparation, in situ hybridization and immunocytochemical detection

The 8-hydroxyquinoline treatment of 3 to 4 day old *Arabidopsis* seedlings did not result in a high number of metaphases in our preparations. A higher number of metaphases were found in young inflorescences (approximately 21 days after germination) or in the secondary flowerbuds 24 h after the main inflorescence (stem = approximately 2.5 cm) had been cut.

We chose to perform the ISH experiments on squash preparations for reasons of accessibility of the target DNA to the biotinylated probe DNA and of the hybrid to the avidin-FITC and biotinylated antibodies. No particular effort was undertaken to strip the preparations of all the cytoplasmic and cell wall material, nor did we try to obtain perfect chromatin or chromosome

spreads. As a result of this many nuclei retained a lot of structure, although this structure, for obvious reasons cannot be regarded as being native.

ISH experiments with the rDNA and the three classes of highly repetitive DNA of *Arabidopsis* have been performed on squash preparations of young inflorescences, stimulated secondary flower buds, leaves of mature plants, 3 to 4 day old seedlings and on protoplast preparations of leaves. The best results were obtained on squash preparations of 3 to 4 day of seedlings. Although some authors report superior results after ISH on protoplasts (Ambros et al. 1986), our ISH experiments on protoplasts were always negative, except after ISH with total genomic DNA (data not shown). From our results it appears that the type and the developmental stage of the tissue are important factors that affect the efficiency of the hybridization and the detection of the hybrids. In this respect it is interesting to note that the hybridization efficiency is influenced by the ultrastructure of the chromatin (Hayashi et al. 1990) and that ISH protocols may have to be adjusted depending on which species is being investigated (Meyne et al. 1990).

The number of metaphases in our preparations was not sufficient, and the cytoplasmic material still too abundant to be able to observe successful ISH with either of the probes on chromosomes. Successful ISH with biotinylated rDNA on metaphase chromosomes of *Arabidopsis* has however already been reported (Murata et al. 1990).

Distribution of the rRNA genes

Table 1 summarizes the results of the ISH experiments with the rDNA probe. Of the 50 nuclei that were studied in detail 34% exhibited 4 labelled sites, 44% less than 4 and 16% more than 4 labelled sites.

Dual wavelength detection of FITC and propidium iodide fluorescence allowed unequivocal localization of the hybridization signals to specific chromocentres.

Figure 1 A and B shows that the most intense hybridization signal was associated with the two largest chromocentres (0.8–1.9 μm in diameter) which were intensely stained with propidium iodide, and were frequently seen to be associated with a large nucleolus (identified as a dark, largely unstained area). These two chromocentres appear to be typical in most of the interphase nuclei of *Arabidopsis*, allowing visual identification of two of the rDNA-associated chromocentres, even without an ISH signal. The one or two smaller hybridization signals were localized to one or two smaller chromocentres (0.5–1.5 μm in diameter), and were less frequently seen to be associated with a nucleolus.. The hybridization signal was frequently seen not to be uniform and suggested two subunits as can be seen from Fig. 1 C.

In a number of cases, strings of spots could be seen emanating from the nucleolus associated chromocentres (NACs). In the nuclei where this was seen in our preparations, these NACs were always the largest two of the four rDNA-associated chromocentres. As can be seen from Fig. 1 D some of the spots located in the nucleolus were clearly labelled. The observations presented in Fig. 1 C and D are in accordance with those made by Rawlins and Shaw (1990) on pea.

Finally, as is shown in Fig. 1 F, a number (eight) of nuclei were seen to exhibit more than four labelled chromocentres. This might be the result of endopolyploidy or of the differential amplification of the rDNA (Nagl 1978).

Table 1. Summary of the results of the in situ hybridization (ISH) experiments with the rRNA genes

	Number of labelled sites per nucleus					
	1	2	3	4	>4	Undetermined
Number of nuclei	0	3	19	17	8	3

In three nuclei the hybridization signal appeared as a diffuse spot

Distribution of the three classes of highly repetitive DNA

The labelling intensity after ISH with any of the three classes of highly repetitive DNA (the 160, 180 or 500 bp repeat respectively) was much less intense than after hybridization with the rDNA. The results were more complicated to interpret since fewer nuclei showed clear hybridization signals and the efficiency of hybridization varied a lot from one experiment to another. Nevertheless, some clear observations could be made from the available data.

The three classes of highly repetitive DNA showed clear differences in hybridization pattern as can be seen from Fig. 2 A–C. The 160 bp repeat presented a characteristic hybridization pattern consisting of three to four pairs of labelled sites and one site that appeared isolated (Fig. 2 A). Some of the labelled sites did not coincide with, but were adjacent to the chromocentres, contrasting with the hybridization pattern of the other two classes of highly repetitive DNA but also with that of the rDNA.

The most important observation that could be made was that although the 180 and the 500 bp repeats hybridized to all the chromocentres [especially clear for the 180 bp repeat (Fig. 2 B)], the distribution of the hybridization signal over the chromocentres was heterogeneous. As can be seen from Fig. 2 C and D, the 500 and 180 bp repeats hybridized more strongly to two chromocentres. This was especially obvious for the 500 bp repeat which was mainly localized on two chromocentres (Fig. 2 C).

ISH experiments with the 180 bp repeat gave the most consistent results regarding hybridization efficiency, and thus allowed the study of the distribution of this class of highly repetitive DNA in the chromatin of the interphase nucleus of *Arabidopsis* in more detail. Results are summarized in Table 2.

Of the 26 nuclei that were looked at more closely 38.5% had a number of labelled chromocentres equal to the total number of chromocentres present in the nucle-

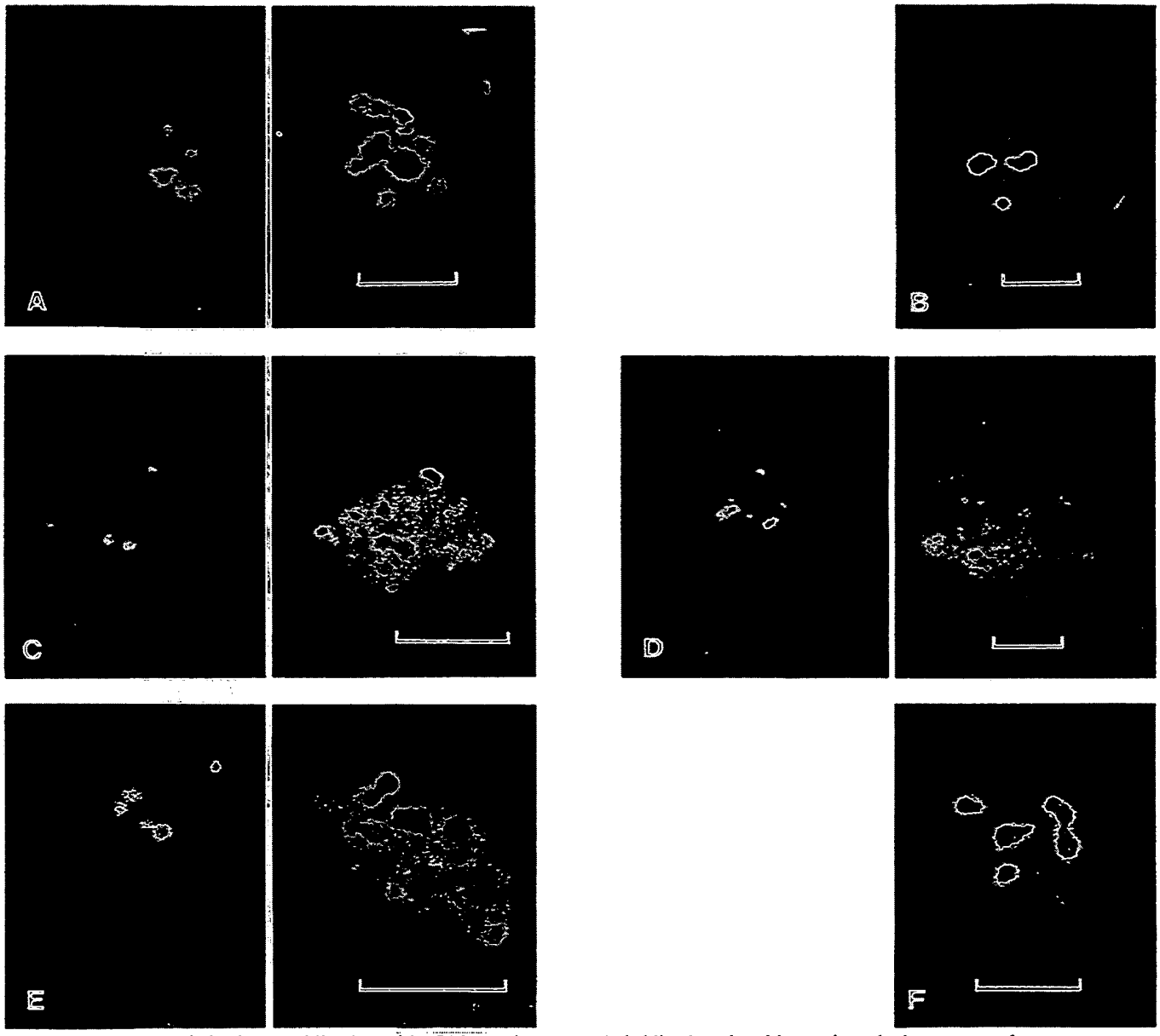
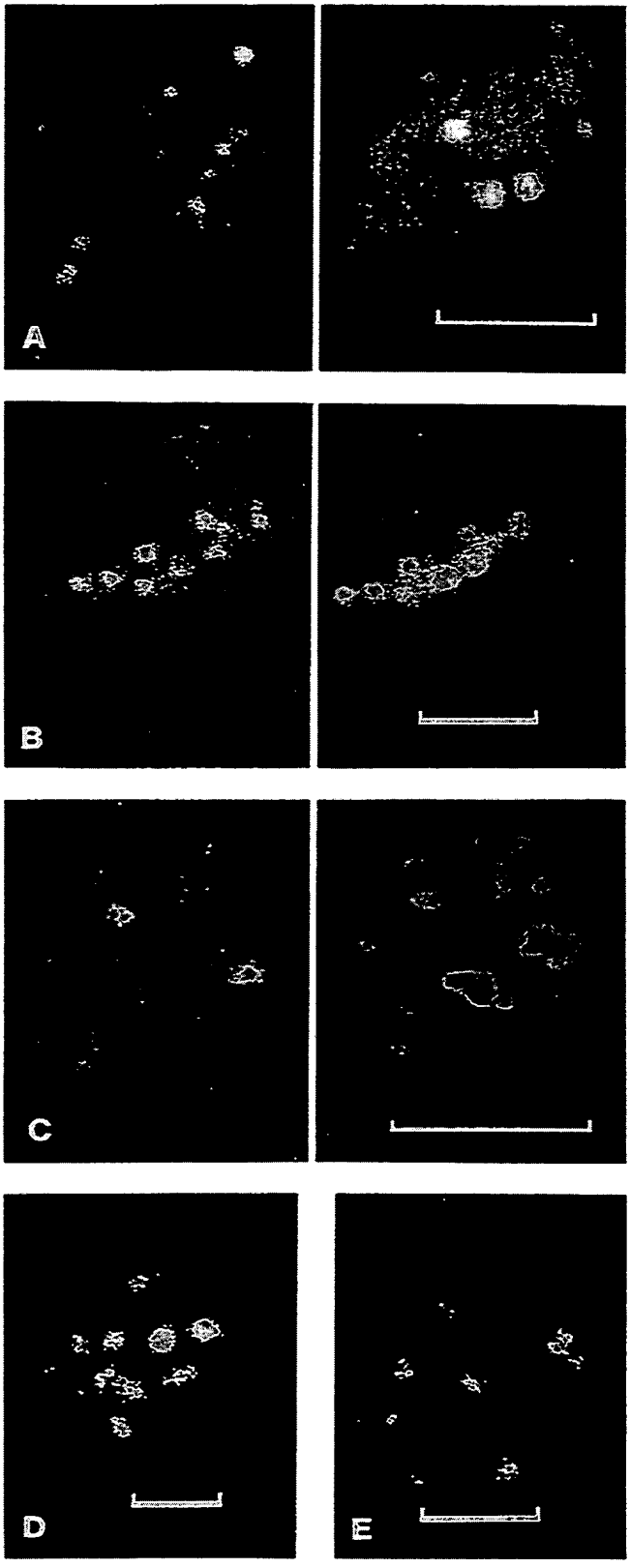


Fig. 1 A–F. Non-isotopic in situ hybridization with rDNA on interphase nuclei of *Arabidopsis*. Hybrids were revealed with fluorescein isothiocyanate (FITC), chromatin was counterstained with propidium iodide and images were recorded by dual wavelength detection with the Bio-rad MRC-500 confocal scanning laser microscopy (CSLM) system. Partial loss of the chromocentre structure of the interphase nuclei may be due to the tissue preparation method. **A** Detection of four sites of rRNA genes in an interphase nucleus (*left*). Counterstained chromatin (*right*). **B** Interphase nucleus showing three locations of rRNA genes (merged image). **C** Interphase nucleus showing four locations of rRNA genes (*left*). The hybridization signal located on the largest two chromocentres suggests the existence of two subunits. Counterstained chromatin (*right*). **D** Detection of sites of rDNA (peri- and intranucleolar) in an interphase nucleus (*left*) presenting a very large nucleolus. Counterstained chromatin (*right*). **E** Localization of rDNA (*left*) on three large nucleolar associated chromocentres in an interphase nucleus. Counterstained chromatin (*right*). One of the three chromocentres clearly shows two locations of rRNA genes. **F** *Arabidopsis* interphase nucleus showing more than four rDNA sites (merged image). Bar represents 5 μm in **A**, **B**, **D** and **F** and 10 μm in **C** and **E**

Table 2. Summary of the results of the ISH experiments with the 180 bp repeat probe

	$N_L = N_T$	$N_L = N_T - 1$	$N_L = N_T - 2$	$N_L = N_T - 3$	Undetermined
Number of nuclei	10	7	3	1	5

N_L, number of labelled chromocentres per nucleus; N_T, total number of chromocentres per nucleus



us. When the total number of chromocentres exceeded the number of labelled ones by one or two (10 nuclei or 38.5%), the one or two unlabelled chromocentres appeared to be the largest present in the interphase nucleus. These are also two of the chromocentres that were identified as being sites where the rDNA is located. In five nuclei either the number of hybridization sites could not be well determined, or the chromocentre structure was not as clear as in the other nuclei.

As was observed for some of the hybridization signals of the rDNA, many chromocentres were not homogeneously labelled after ISH with the 180 bp repeat probe. Figure 2E shows that two or more labelled spots could be seen to be localized on many chromocentres. It should also be noted that the two chromocentres that were seen to be more intensely labelled after ISH with the 180 bp repeat probe were not the largest two present in the interphase nucleus. Interestingly, when the two largest chromocentres, present in most of the interphase nuclei, were seen to be labelled after ISH with the 180 bp repeat probe, the hybridization signal sometimes appeared as a band over, or at the edge of, the chromocentre.

Discussion

Fluorescence microscopy

The Bio-rad MRC-500 CSLM system was used for recording images of microscopical preparations. The use of squash preparations makes the application of the optical sectioning possibility of the confocal microscope superfluous, but the digital fluorescence imaging properties of the instrument enhance the accuracy of observation.

The Bio-rad MRC-500 CSLM is fitted with (amongst others) a filter-combination that very effectively separates the fluorescence light emitted by FITC (green) and propidium iodide (red) respectively. This allows the re-

Fig. 2A–E. Non-isotopic in situ hybridization with three classes of highly repetitive DNA on interphase nuclei of *Arabidopsis*. Hybrids were revealed with FITC, chromatin was counterstained with propidium iodide and images were recorded by dual wavelength detection with the Bio-rad MRC-500 CSLM system. Partial loss of the chromocentre structure of the interphase nuclei may be due to the tissue preparation method. **A** Hybridization pattern after ISH with the 160 bp repeat on an interphase nucleus (*left*). Counterstained chromatin (*right*). **B** Hybridization pattern after ISH with the 180 bp repeat on an interphase nucleus (*left*). All chromocentres are clearly labelled. Counterstained chromatin (*right*). **C** Hybridization pattern after ISH with the 500 bp repeat on an interphase nucleus (*left*). Two chromocentres are intensely labelled. Counterstained chromatin (*right*). **D** Two chromocentres in an interphase nucleus can be seen to be more intensely labelled after ISH with the 180 bp repeat (merged image). **E** The chromocentres in an interphase nucleus can be seen to be unevenly labelled after ISH with the 180 bp repeat, suggesting two or more subunits (merged image). Bar represents 10 μm in **A**, **B**, **C** and **E** and 5 μm in **D**

cording of a faint ISH signal against a background of counterstained chromatin. Moreover, detection of the two signals is not sequential but simultaneous, which allows a very precise localization of the ISH signal in the chromatin or on metaphase chromosomes.

Also, although the detector diaphragm of the green channel (the ISH signal) of the CSLM was fully opened, resulting in the loss of the optical sectioning property, the point excitation with the scanning laser nevertheless results in an enhanced image quality (Shotton 1989).

Distribution of the rRNA genes

Our results confirm the results obtained by Murata et al. (1990) on metaphase chromosomes. These authors observed two pairs of nucleolar chromosomes, tentatively identified as chromosomes 2 and 4. A clear difference in the size of the signals between the two pairs of nucleolar chromosomes was observed.

Four labelled chromocentres were clearly observed in 34% of the nuclei, two of which were clearly more intensely labelled than the other two. This has also been observed in *Pisum sativum*, which also has two pairs of rDNA-bearing chromosomes (Rawlins and Shaw 1990). The fact that in 38% of the nuclei only three, and in 6% only two chromocentres were observed can be accounted for in a number of ways. First, it may be that the four sites are not always detected because of the fact that the hybridization efficiency is not 100%. This is however not very plausible, since the intranuclear variation is very low for ISH (Nederlof et al. 1989a). Second, one labelled chromocentre might hide another in the flattened nucleus. A third possibility may be the somatic association of active nucleolus organizing regions (NORs) to form a reduced number of rDNA-bearing chromocentres, associated with a nucleolus. The phenomenon of NORs moving within the nucleus, and associating, depending on their transcriptional activity, to form a reduced number of sites labelled after ISH with rDNA, has been observed in phytohaemagglutinin-stimulated human lymphocytes (Wachtler et al. 1986). Specific associations between NOR-bearing chromosomes have also been described in a number of cases (reviewed by Appels 1989).

We have found statistical evidence that the number of chromocentres in the interphase nucleus is correlated with the average size of the chromocentres (data not shown), indicating that there may be somatic association of chromosomes in the interphase nucleus. Comparison between the number of labelled chromocentres and the total number of chromocentres in the nucleus did not however yield any conclusive evidence of NORs moving to form a reduced number of labelled chromocentres.

Although it should be borne in mind that the ISHs were performed on squash preparations, striking differences in location between the two pairs of NORs could be observed as can be seen from Figs. 1A, C and E. In a number of nuclei three large labelled chromocentres were seen to be associated with a large nucleolus, indicating that the two smaller NORs might have paired to form a larger active NAC. As can be seen from Fig. 1E one of the three chromocentres clearly carries two labelled areas.

Although the statistical evidence is not conclusive, morphological observations would plead for NORs moving and associating to form a reduced number of labelled chromocentres, their activation being a result of this displacement (Appels 1989; Gross and Garrard 1988).

Table 3. Number of copies and number of base pairs (bp) per haploid genome for each of the three classes of highly repetitive DNA and the rDNA

DNA	%[a]	N[b]	bp ($\times 10^6$)[c]
160 bp repeat	0.2–0.4	1790– 3580	0.286–0.572
180 bp repeat	0.8–1.4	6400–11100	1.15–2.00
500 bp repeat	0.2–0.4	570– 1140	0.286–0.572
rDNA[d]	5.7	570	5.7

[a] Percentage of total cellular DNA (Simoens et al. 1988)
[b] Copy number per haploid genome
[c] Number of base pairs per haploid genome
[d] Meyerowitz and Pruitt (1985)

Distribution of the three classes of highly repetitive DNA

The three classes of highly repetitive DNA used in the ISH experiments consisted of a 160, a 180 and a 500 bp repeat, arranged in tandem arrays (Simoens et al. 1988). Taking into account the recently revised size of the *Arabidopsis* nuclear genome [100×10^6 bp per haploid genome (Heslop-Harrison and Schwarzacher 1990) instead of 70×10^6 (Meyerowitz and Pruitt 1985)] and the fact that up to 30% of total cellular DNA can be chloroplast DNA (Leutwiler et al. 1984) one can estimate their respective copy number. The revised copy numbers are listed in Table 3.

The rRNA genes of *Arabidopsis* are largely arranged in tandem arrays. Considering that these tandem arrays are spread (unevenly) over only four loci, it is obvious why the intensity of the ISH signal detected for each of the three classes of highly repetitive DNA was consistently lower than that of the rDNA. This could also be a reason why the results were less consistent from one ISH experiment to another for the three classes of highly repetitive DNA as compared with the rDNA, the hybridization signal being intense enough in the case of the rDNA, to be detected even at strongly reduced efficiency of hybridization. This was especially true for the 160 bp repeat, a sequence apparently quite evenly spread over nine loci.

Some regions of the 500 bp repeat show 70%–80% homology with the 180 bp repeat. The 500 bp repeat presumably arose by duplication of one-half of a 180 bp repeat and insertion of a foreign element between the two duplicated parts (Simoens et al. 1988). Thus, cross hybridization must have occurred.

The question of whether the two chromocentres that were more intensely labelled after ISH with the 180 bp repeat (Fig. 2D) or the 500 bp repeat (Fig. 2C) reflect the presence of larger cluster of 180 bp repeat units and 500 bp repeat units on two different pairs of homologous chromosomes, or the presence on the same pair of homologues of large clusters of 180 bp and 500 bp repeat units, or are simply the reflection of cross hybridization of the 180 bp repeat with a larger cluster of 500 bp repeat units on one pair of homologues, should be answered by two sets of experiments: a double labelling non-isotopic ISH experiment with the 180 bp and the 500 bp repeat, and an ISH experiment in which labelled 500 bp repeat probe DNA is mixed with unlabelled 180 bp repeat DNA to suppress cross hybridization (Pinkel et al. 1988). It should be noted that in each of the three cases the 500 bp repeat could be used as a chromosome specific repetitive DNA probe since the difference in labelling intensity is the largest in the case of the 500 bp repeat.

Multiple labelling experiments involving the 160 bp repeat might also yield interesting information about the relative positions of the different classes of highly repetitive DNA in *Arabidopsis*, since it was seen that some of the hybridization sites of this repeat were localized outside the chromocentres in the interphase nuclei we analysed. The importance of multiple labelling experiments on interphase nuclei has already been demonstrated (Nederlof et al. 1989a; Lichter and Ward 1990). It is of particular interest in the case of *Arabidopsis*, where good metaphase spreads are particularly hard to come by.

As a final remark it is important to bear in mind that the nuclei that were studied in each of the four ISH experiments represented a random sample of cell nuclei from different tissues of a 3 to 4 day old *Arabidopsis* seedling. The differences in distribution of the labelled sites that were observed, especially for the rDNA, almost certainly reflect cell cycle and tissue specific differences in the distribution of the DNA sequences. Further refinements in the ISH technique (tissue preservation, sensitivity, quantitative analysis) will allow the study of the cell cycle and tissue specific localization of repetitive and single copy genes in the interphase nucleus, and will allow the correlation of these data with the level of expression of the genes concerned.

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References

Agard DA, Hiraoka Y, Shaw P, Sedat JW (1989) Fluorescence microscopy in three dimensions. Methods Cell Biol 30B:353–377

Ambros PF, Matzke MA, Matzke AJM (1986) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 94:11–18

Appels R (1989) Three-dimensional arrangements of chromatin and chromosomes: old concepts and new techniques. J Cell Sci 92:325–328

Arndt-Jovin DJ, Jovin TM (1989) Fluorescence labelling and microscopy of DNA. Methods Cell Biol 30B:417–448

Bauwens S, Van Oostveldt P (1990) Microfluorometric measurement of the relative amount of DNA in plant cell nuclei. Protection against photo-bleaching – Confocal observations. Med Fac Landbouww Rijksuniv Gent 53(3b):1229–1240

Brakenhoff GJ, van Spronsen EA, van der Voort HTM, Nanninga N (1989) Three-dimensional confocal fluorescence microscopy. Methods Cell Biol 30B:379–398

Dyer AF (1979) In: Investigating chromosomes. Edward Arnold, London, p 24

Elgin SCR (1990) Chromatin structure and gene activity. Curr Opin Cell Biol 2:437–445

Gall JG (1981) Chromosome structure and the C-value paradox. J Cell Biol 91:3s–14s

Gross DS, Garrard WT (1988) Nuclease hypersensitive sites in chromatin. Annu Rev Biochem 57:159–197

Hayashi S, Ruddell A, Sinclair D, Grigliatti T (1990) Chromosomal structure is altered by mutations that suppress or enhance position effect variegation. Chromosoma 99:391–400

Heslop-Harrison JS, Schwarzacher T (1990) The ultrastructure of *Arabidopsis thaliana* chromosomes. In: Schweizer D, Peuker K, Loidl J (eds) Fourth International Conference on Arabidopsis Research, Vienna, June 2–5, Abstracts, p 3

Hiraoka Y, Sedat JW, Agard D (1987) The use of charge-coupled device for quantitative optical microscopy of biological structures. Science 238:36–41

Hiraoka Y, Minden JS, Swedlow JR, Sedat JW, Agard DA (1989) Focal points for chromosome condensation and decondensation revealed by three-dimensional in vivo time-lapse microscopy. Nature 342:293–296

Hochstrasser M, Sedat JW (1987a) Three-dimensional organization of *Drosophila melanogaster* interphase nuclei. I. Tissue-specific aspects of polytene nuclear architecture. J Cell Biol 104:1455–1470

Hochstrasser M, Sedat JW (1987b) Three-dimensional organization of *Drosophila melanogaster* interphase nuclei. II. Chromosome spatial organization and gene regulation. J Cell Biol 104:1471–1483

Hochstrasser M, Mathog D, Gruenbaum Y, Saumweber H, Sedat JW (1986) Spatial organization of chromosomes in the salivary gland nuclei of *Drosophila melanogaster*. J Cell Biol 102:112–123

Leutwiler LS, Hough-Evans BR, Meyerowitz EM (1984) The DNA of *Arabidopsis thaliana*. Mol Gen Genet 194:15–23

Lichter W, Ward DC (1990) Is non-isotopic in situ hybridization finally coming of age? Nature 345:93–95

Manuelidis L, Borden J (1988) Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three-dimensional reconstruction. Chromosoma 96:397–410

Martinez-Zapatar JM, Estelle MA, Somerville CR (1986) A highly repeated DNA sequence in *Arabidopsis thaliana*. Mol Gen Genet 240:417–423

Meyerowitz EM, Pruitt RE (1985) *Arabidopsis thaliana* and plant molecular genetics. Science 229:1214–1218

Meyne J, Baker RJ, Hobart HH, Hsu TC, Ryder OA, Ward OG, Wiley JE, Wurster-Hill DH, Yates TL, Moyzis RK (1990) Distribution of non-telomeric sites of the $(TTAGGG)_n$ telomeric sequence in vertebrate chromosomes. Chromosoma 99:3–10

Murata M, Varga F, Maluszynska J, Gruendler P, Schweizer D (1990) Chromosomal localization of the ribosomal RNA genes in *Arabidopsis thaliana* by in situ hybridization. In: Schweizer D, Peuker K, Loidl J (eds) Fourth International Conference on Arabidopsis Research, Vienna, June 2–5, Abstracts, p 5

Nagl W (1978) Mechanisms of endopolyploidization. In: Nagl W (ed) Endopolypoidy and polyteny in differentiation and evolution. Elsevier, Amsterdam, pp 66–113

Nagl W (1979) Condensed interphase chromatin in plant and animal cell nuclei: fundamental differences. Plant Syst Evol [suppl] 2:247–260

Nederlof PM, Robinson D, Abuknesha J, Wiegant J, Hopman AHN, Tanke HJ, Raap AK (1989a) Three-color fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. Cytometry 10:20–27

Nederlof PM, Van der Flier S, Raap AK, Tanke HJ, Van der Ploeg M, Kornips F, Geraerdts JPM (1989b) Detection of chromosome aberrations in interphase tumor nuclei by nonradioactive in situ hybridization. Cancer Genet Cytogenet 42:87–98

Oud JL, Mans A, Brakenhoff GJ, van der Voort HTM, van Spronsen EA, Nanninga N (1989) Three-dimensional chromosome arrangement of *Crepis capillaris* in mitotic prophase and anaphase as studied by confocal scanning laser microscopy. J Cell Sci 92:329–339

Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J (1988) Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci USA 85:9138–9142

Rawlins DJ, Shaw PJ (1990) Three-dimensional organization of ribosomal DNA in interphase nuclei of *Pisum sativum* by in situ hybridization and optical tomography. Chromosoma 99:143–151

Schwarzacher T, Ambros P, Schweizer D (1980) Application of Giemsa banding to orchid karyotype analysis. Plant Syst Evol 134:293–297

Shotton DM (1989) Confocal scanning optical microscopy and its applications for biological specimens. J Cell Sci 94:175–206

Simoens CR, Gielen J, Van Montagu M, Inzé D (1988) Characterization of highly repetitive sequences of *Arabidopsis thaliana*. Nucleic Acids Res 16:6753–6766

Unfried I, Gruendler P (1990) Nucleotide sequence of the 5.8 S and 25 S rRNA genes and of the internal transcribed spacers from *Arabidopsis thaliana*. Nucleic Acids Res 18:4011

Unfried I, Stocker U, Gruendler P (1989) Nucleotide sequence of the 18 S rRNA gene from *Arabidopsis thaliana* Co 10. Nucleic Acids Res 18:7513

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA 85:5536–5540

Van Dekken H, Pinkel D, Mullikin J, Trask B, van den Engh G, Gray J (1989) Three-dimensional analysis of the organization of human chromosome domains in human and human-hamster interphase nuclei. J Cell Sci 94:299–306

Wachtler F, Hopman AHN, Wiegant J, Schwarzacher HG (1986) On the position of the nucleolus organizer regions (NORs) in interphase nuclei. Studies with a new, non-autoradiographic in situ hybridization method. Exp Cell Res 167:227–240

Chromosomal distribution of a repeated DNA sequence from C-genome heterochromatin and the identification of a new ribosomal DNA locus in the *Avena* genus

Araceli Fominaya, Gregorio Hueros, Yolanda Loarce, and Esther Ferrer

Abstract: Satellite DNA specific to the oat C genome was sequenced and located on chromosomes of diploid, tetraploid, and hexaploid *Avena* ssp. using in situ hybridization. The sequence was present on all seven C genome chromosome pairs and hybridized to the entire length of each chromosome, with the exception of the terminal segments of some chromosome pairs. Three chromosome pairs belonging to the A genome showed hybridization signals near the telomeres of their long arms. The existence of intergenomic chromosome rearrangements and the deletions of the repeated units are deduced from these observations. The number of rDNA loci (18S–5.8S–26S rDNA) was determined for the tetraploid and hexaploid oat species. Simultaneous in situ hybridization with the satellite and rDNA probes was used to assign the SAT chromosomes of these species to their correct genomes.

Key words: oats, satellite DNA, rDNA, in situ hybridization, genome evolution.

Résumé : Un ADN satellite spécifique au génome C de l'avoine a été séquencé. La distribution de cette séquence chez les chromosomes d'espèces diploïdes, tétraploïdes et hexaploïdes du genre *Avena* a été déterminée par hybridation in situ. La séquence est présente sur chacun des sept chromosomes du génome C et l'hybridation était visible sur toute la longueur des chromosomes à l'exception des régions terminales de quelques chromosomes. Trois chromosomes appartenant au génome A ont montré de l'hybridation à proximité des télomères de leurs bras longs. L'existence de réarrangements chromosomiques intergénomiques et les délétions des unités répétées sont déduites de ces observations. Le nombre de loci d'ADN ribosomique (18S–5.8S–26S ADNr) a été déterminé chez les espèces tétraploïdes et hexaploïdes d'avoine. L'hybridation in situ simultanée avec des sondes d'ADN satellite et d'ADNr a été employée afin d'assigner les chromosomes SAT de ces espèces aux génomes appropriés.

Mots clés : avoine, ADN satellite, ADNr, hybridation in situ, évolution des génomes.
[Traduit par la Rédaction]

Introduction

Avena eriantha Dur. (*A. pilosa* M. Bieb.), *A. clauda* Dur., and *A. ventricosa* Bal. are diploid C-genome species with a centre of variation extending throughout the Mediterranean basin and the Middle East to Afghanistan (Leggett 1992). Diploid species with the C-genome are known to be involved in the parentage of the tetraploid oats *A. maroccana* Gdrg. and *A. murphyi Ladiz.* ($2n = 4x = 28$, genome composition AACC), the hexaploid oats *A. fatua* and *A. sterilis*, and the cultivated species *A. byzantina* and *A. sativa* ($2n = 6x = 42$, genome composition AACCDD). In addition to their phylogenetic importance, species with the C-genome might also be an important resource for improving the genetic variability of cultivated oats.

Over the past few years, cytogenetic and isoenzymatic markers have been used as a means of gaining insight into the relationships between the A and C genomes of the wild *Avena* species and the A, C, and D genomes of cultivated oats. The first genomic relationships found were based on the degree of chromosome pairing observed in F_1 interspecific hybrids (Rajhathy and Thomas 1974). C-banding techniques made it possible to describe the heterochromatin patterns of the three constituent genomes of hexaploid oats (Fominaya et al. 1988*a*, 1988*b*; Linares et al. 1992; Jellen et al. 1993*a*,

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A. Fominaya, G. Hueros, Y. Loarce, and E. Ferrer. Department of Cell Biology and Genetics, University of Alcalá de Henares, Campus Universitario, E-28871 Alcalá de Henares, Madrid, Spain.

Table 1. Plant materials used in this study, their nomenclature and sources.

Species	Genome	Accession	Sources
A. eriantha	CC	CAV 0063	Dr. R. Loiselle, Plant Research Centre, Ottawa, Ontario, Canada
A. clauda	CC	CAV 0001	Dr. R. Loiselle, Plant Research Centre, Ottawa, Ontario, Canada
A. maroccana	AACC	CAV 4388	Dr. R. Loiselle, Plant Research Centre, Ottawa, Ontario, Canada
A. murphyi	AACC	CAV 2832	Dr. R. Loiselle, Plant Research Centre, Ottawa, Ontario, Canada
		CC 7120	Dr. M.D. Leggett, Welsh Plant Breeding Station, Great Britain
A. byzantina	AACCDD	Cv. Kanota	Dr. T. Morikawa, University of Osaka, Prefecture, Japan
A. sterilis	AACCDD	PI 411958	Dr. H. Smith, National Small Grain Collection, Beltsville, Md., U.S.A.
A. sativa	AACCDD	Cv. Pandora Cv. Prevision	National Institute of Seeds, Madrid, Spain

1993*b*). These studies characterized C-genome chromosomes by their strong heterochromatic bands at the centromeric and interstitial regions, while A- and D-genome chromosomes were characterized by a small number of heterochromatic bands at the telomeric or interstitial regions. Chromosome rearrangements were proposed to explain several features of the heterochromatin distribution of these species. The patterns of isoenzymatic variation in 12 species of oats with different ploidy levels suggested that *A. murphyi* could be implicated in the evolution of the hexaploid oats (Sanchez de la Hoz and Fominaya 1989).

Relationships between plant species have been established by studying repetitive DNA sequences. These sequences account for a very large part of a plant genome and are mainly responsible for the variation in genome size among different species of the same genus (Flavell 1982; Walbot and Cullis 1985). Specific repetitive DNA sequences, both tandemly arranged and dispersed throughout the genome, have been reported in a large number of plant species, including oats (Fabijanski et al. 1990; Solano et al. 1992; Gupta et al. 1992). Although their function and origin remain unknown, the repetitive sequences change rapidly on an evolutionary time scale in comparison with coding sequences (Singer 1982; Flavell 1982). This feature makes it possible to isolate repetitive sequences that are specific to a taxonomic group, i.e., tribe, genus, or species, and which, therefore, are useful for studying genome evolution at the molecular level (McIntyre et al. 1988; Zhao et al. 1989; Tsujimoto and Gill 1991; Kenton et al. 1993).

In a previous paper (Solano et al. 1992), a repetitive DNA sequence from *A. murphyi*, Am1, was described. This was present in species with the C genome and absent in diploid and tetraploid species with A and B genomes. Restriction and Southern hybridization analyses showed that the sequence was tandemly arranged, and the length of the repeated unit was estimated as 58 base pairs (bp). These features indicated that the sequence might be satellite DNA. Since this kind of sequence seems to be located mainly on the heterochromatic regions of the chromosomes (John and Miklos 1979), analysis of the chromosome distribution of the Am1 sequence in oat species could help to elucidate the origin of the C-banding patterns seen in the oat chromosomes. Also, the possibility of using Am1 as a genome marker could be exploited for chromosome and genome identification of defined DNA sequences using simultaneous in situ hybridization. C-banding and Ag-NOR (silver staining of NORs) techniques have proved invaluable for identifying satellited chromosomes in tetraploid and hexaploid oats (Fominaya et al. 1988*a*, 1988*b*; Linares et al. 1992; Jellen et al. 1993*a*, 1993*b*). The ribosomal genes of hexaploid oats have recently been mapped to the secondary constrictions of six chromosomes using radioactive in situ hybridization (Jellen et al. 1994*a*). Simultaneous fluorescence in situ hybridization with Am1 and NOR sequences ought to give better resolution, and thus a more precise mapping of the signals might be obtained. The aim of the present paper was to verify the satellite nature of the Am1 sequence and to study its distribution on the chromosomes of oat species with the C genome using in situ hybridization. Further, the localization of the ribosomal sequences to specific chromosomes in tetraploid and hexaploid species of *Avena* was also undertaken in order to assign them to genomes.

Materials and methods

Plant materials and root-tip preparations

The names of the species employed in this study, their accession numbers, and sources are listed in Table 1. Nomenclature and genome designation are based on the work of Rajhathy and Thomas (1974). Seeds were germinated on moist filter paper for 24 h at 25°C, 36 h at 4°C, and then 26 h at 25°C, in order to synchronize cell divisions. The seedlings were then transferred to ice water for 28 h at 0°C to accumulate metaphases before fixation in ethanol – acetic acid (3:1).

DNA probes

The probe pAm1 consisted of plasmid pUC19 containing a 464 bp *Bam*HI fragment isolated from *A. murphyi* DNA. The fragment hybridized with repetitive DNA specific to the C genome of oats (Solano et al. 1992). The probe pTA71 contained a 9 kilobase (kb) *Eco*RI fragment, including the 18S–5.8S–26S rDNA isolated from wheat, *Triticum aestivum* (Gerlach and Bedbrook 1979).

Genome copy numbers of Am1 sequences

Genomic DNA from *A. clauda*, *A. maroccana*, and *A. sativa* and the plasmid pAm1 were serially diluted and blotted onto a Zeta-Probe membrane (BioRad), according to the manufacturer's instructions. The insert of pAm1 was labelled with digoxigenin-11-dUTP (Boehringer Manheim) by polymerase chain reaction (PCR). Hybridization, washes, and chemiluminescent detection were essentially as described by Hoisington (1992). Hybridization signals were quantified using a Millipore Bio Image. Copy numbers in the different species were calculated by comparing signal intensities between genomic and plasmid dilutions. The genome sizes of the *Avena* species were taken from Bennett and Smith (1976).

DNA sequencing

The nucleotide sequence of a fragment of 313 bp from the insert cloned in pAm1 was determined by the dideoxy termination method using double-stranded plasmid templates with T7 DNA polymerase (Sequenase Version No. 2.0, U.S. Biochemicals).

Chromosome preparation

Cytological preparations for in situ hybridization were made as described by Maluszynska and Heslop-Harrison (1993), with some modifications. Prior to squashing, root tips were washed in 0.01 M citric acid – sodium citrate (ph 4.8) for 30 min and digested in 2% (w/v) cellulase (Calbiochem) in 20% (v/v) pectinase (Sigma) for 1.5 h at 37°C. Meristemic regions were then excised, and one root tip per slide was squashed in a drop of 45% acetic acid. The cover slip was removed by freezing the slides and the preparation was air-dried. Slide preparations were incubated in 100 μg/mL DNase-free RNase in 2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) for 1 h at 37°C and washed three times in 2× SSC for 5 min. The slides were post-fixed in freshly depolymerized 4% (w/v) paraformaldehyde in water for 10 min, washed in 2× SSC for 15 min, dehydrated in a graded ethanol series, and air-dried.

Labelling of DNA probe

The whole plasmids of pAm1 and pTa71 were labelled by nick translation with biotin-14-dATP (Gibco) and digoxigenin-11-dUTP, respectively. Also, the whole plasmid of pAm1 was labelled with rhodamine-4-dUTP (Amersham) by PCR. All probes were precipitated with ethanol.

In situ hybridization

The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulphate), 2× SSC, 10 ng of sheared and denatured salmon sperm DNA, and 3 μg/mL of each DNA probe (combining both pAM1 and pTa71 probes, each fluorescence labelled with a different system when necessary). The mixture was denatured for 15 min. Thirty microlitres of this mixture was loaded onto the slide preparation and covered with a plastic coverslip. The slides were then placed in a modified programmable temperature controller, denatured for 7 min at 75°C, and incubated overnight at 37°C in a humidity chamber (Heslop-Harrison et al 1991).

Posthybridization washes and detection

Following hybridization, the hybridized slides with biotinylated probes were washed in 2× SSC at room temperature for 5 min, 2× SSC at 37°C for 10 min, 2× SSC at room temperature for 5 min, 0.2% Triton X-100 in phosphate-buffered saline (PBS) at room temperature for 5 min, and PBS at room temperature for 1 min. For detection of hybridization sites, the slides were treated with 120 μL of a solution of a complex of streptavidin – biotinylated horseradish peroxidase in detection buffer. Slides were covered with plastic coverslips and incubated at 37°C for 1 h. After removal of the coverslips, the slides were washed in 2× SSC for 5 min, 0.2% Triton X-100 in PBS for 2 min, and PBS for 1 min, at room temperature. For visualization of the hybridization sites, 450 μL of a solution of 0.05% diaminobenzidine tetrahydrochloride (Sigma) in 0.01 M Tris–HCl buffer, pH 7.4, and 0.015% H_2O_2 were placed on the slides at 4°C for 20 min. The slides were rinsed with PBS, counterstained with 2% Giemsa (Gurr) for 1 min, air-dried, and mounted with DepeX. Slides were examined with a Zeiss microscope and photographs were taken using Kodak Imagelink HQ film ASA 12.

The hybridized slides with rhodamine and digoxigenin probes were washed once in 2× SSC for 5 min at 42°C and then given a stringent wash in 20% formamide in 0.1× SSC for 10 min at 42°C. The slides were washed in 0.1× SSC for 5 min at 42°C, 2× SSC for 5 min at 42°C, and transferred to detection buffer (4× SSC, 0.2% Tween 20) for 5 min. To detect digoxigenin labelled probes, each slide was treated with sheep anti-digoxigenin-fluorescein (FITC) (Boehringer Mannheim). Slides were treated with 5% (w/v) BSA in detection buffer for 5 min, and then incubated in a solution of 1:40 FITC in detection buffer containing 5% (w/v) BSA for 1 h at 37°C. No immunocytochemistry was necessary to detect the rhodamine labelling after posthybridization washes. All slides were washed three times in detection buffer for 10 min at room temperature, counterstained with 2 μg/mL 4,6-diamidino-2-phenylindole (DAPI) in McIlvaine's citrate buffer, pH 7.0, and then mounted in antifade solution (A F1, Citifluor). Slides were examined with a Zeiss Axiophot epifluorescence microscope with Zeiss filter sets: 01 for DAPI, 09 for fluorescein, and 15 for rhodamine. Photographs were taken using Fujicolor super G 400 color print film.

Results

Sequence analysis of Am 1

The nucleotide sequences of six monomers cloned in the plasmid pAm1 were determined (Fig. 1). The consensus sequence was 51 bp long with a A + T content of 58%. No relevant internal direct repeats were detected, though an

imperfect inverted repeat of about 14 bp was found in the consensus sequence. The nucleotide sequences of the inverted repeats shared 68% homology with each other and could form hairpin structures. The nucleotide sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession Number X83958.

Five of the six monomer sequences were remarkably conserved, showing an average of more than 92% nucleotide sequence similarity. The number of base substitutions (8) was similar to the number of deletion–additions (5), and all of them appeared to be randomly spread over the whole sequence. The nucleotide sequence from unit 2 in Fig. 1 was rather divergent from the consensus. It presented a 12 bp deletion at the beginning of the unit, an 8 bp addition, and 7 base substitutions spread over the sequence. A search of the EMBL sequence data bank found no homology with any previously published sequence.

Fig. 1. Sequence of a 313 bp fragment cloned in pAm1 that includes six complete monomers. The consensus sequence has been written on the first line. Nucleotides common to the consensus are indicated by hyphens; sequence differences are denoted by the relevant nucleotide, deletions are indicated by dots, and insertions are written under the sequence. The two imperfect inverse repeats are overlined.

```
                5'----------------------3'

C GATCCATGTGTGGTTTGTGGAAAGAACACACATGCAATGACTCTAGTGGTT
1 G-----------------------------------------------C-A
2 ............G-------T--C-C--A-------------AG-------
                TTTTAGTA
3 G-----------------------------------------T----------
4 G--------------------------------------..-C-C----G
5 G-----------------------------------------------------
6 G -------.-T----------T-------------------------------
7 G------------
```

Abundance of Am1 in the oat genomes

The abundance of the Am1 sequence in the three oat species that represented all the ploidy levels found in the genus was investigated. Copy numbers of 1.4×10^5, 1.3×10^5, and 9.2×10^4 were obtained for *A. clauda*, *A. maroccana*, and *A. sativa*, respectively. These figures indicated that no gross deletions of this repetitive DNA had accompanied the evolution of these species, although a loss of copies seemed to have occurred in the transition from the tetraploid to the hexaploid. The Am1 family was, therefore, considered to be a highly repetitive DNA family in these species. The features so far described for the Am1 sequence indicate that it is a satellite DNA sequence.

Chromosomal distribution of Am1 tandemly repeated sequences

The karyotype of diploid and tetraploid species was described by Rajhathy and Thomas (1974). Fominaya et al. (1988*a*, 1988*b*), using C-banding and silver nitrate staining of nucleolus organizer regions (NOR), studied the karyotypes in greater detail. The nomenclature used in the above papers is followed here to designate the C-genome chromosomes of these species. Rajhathy and Thomas (1974) and Morikawa (1985) reported the hexaploid karyotype and numbered, from 1 to 21, each chromosome pair on the basis of their overall length and arm ratios. Using this chromosome classification, Linares et al. (1992), with information obtained by C-banding, allocated individual chromosomes to specific genomes. These designations will be used for the chromosomes of the hexaploid species.

To determine the organization and distribution of this newly discovered repeated sequence family on the chromosomes, pAm1 was used as a probe in in situ hybridization. Figures 2 and 3 show in situ hybridization patterns of the species *A. eriantha*, *A. murphyi*, *A. maroccana*, *A. sativa*, and *A. sterilis*, using pAm1 as probe. When fluorescence in situ hybridization (FISH) was used, the hybridization sites were detected as red fluorescence (rhodamine). Nonhybridizing chromosomal sequences fluoresced blue (DAPI) (Fig. 2). In contrast, when the detection of hybridization was carried out enzymatically with streptavidin – horseradish peroxidase, pAm1 hybridization sites appeared as brown regions, whereas nonhybridizing chromosomal sequences appeared blue owing to Giemsa counterstaining (Fig. 3).

The 464 bp insert of pAm1 hybridized strongly and evenly over the entire length of all chromosomes of *A. eriantha* and *A. clauda* (Figs. 2*a* and 2*b*). When pAm1 was hybridized with *A. murphyi* metaphases, two sets of chromosomes, each with 14 chromosomes, were clearly identified (Figs. 2*c*–2*f*). The hybridization patterns of one of the chromosome sets were like those of the diploid C genome species. This chromosome set was consequently assigned to the C-genome chromosomes. The remaining 14 chromosomes, which were almost unhybridized, were identified as A-genome chromosomes. However, differences in the hybridization patterns were observed within each chromosome set. Thus, within the C genome, three chromosome pairs showed uniform red fluorescence, indicating their overall hybridization to the probe. The chromosomes were identified as 2C, 5C, and 10C on the basis of overall length and arm ratio. Three pairs of chromosomes from the C genome were partially hybridized, fluorescing red with rhodamine, except for short segments of DAPI fluorescence on their long arms. Karyotyping data identified these chromosome pairs as 1C, 6C, and 9C. The last chromosome pair from the C genome was partially hybridized, fluorescing red over both arms, except for a short segment of DAPI fluorescence on the short arm. This chromosome was identified as 3C. Regarding the A-genome chromosomes, three chromosome pairs were partially hybridized, fluorescing blue with DAPI, except for a short segment of red fluorescence in the nearly telomeric regions of the long arms. These chromosomes were identified as 8A, 12A, and 13A. The remaining four chromosome pairs from the A genome were unhybridized, fluorescing blue with DAPI over the length of the chromosomes.

Similar results were found after hybridization of pAm1 to metaphase plates of *A. maroccana* (Figs. 2*g*–2*h*). In this case, four chromosome pairs from the C genome showed strong hybridization in both arms. Three red chromosome pairs from the C genome containing blue nonhybridizing

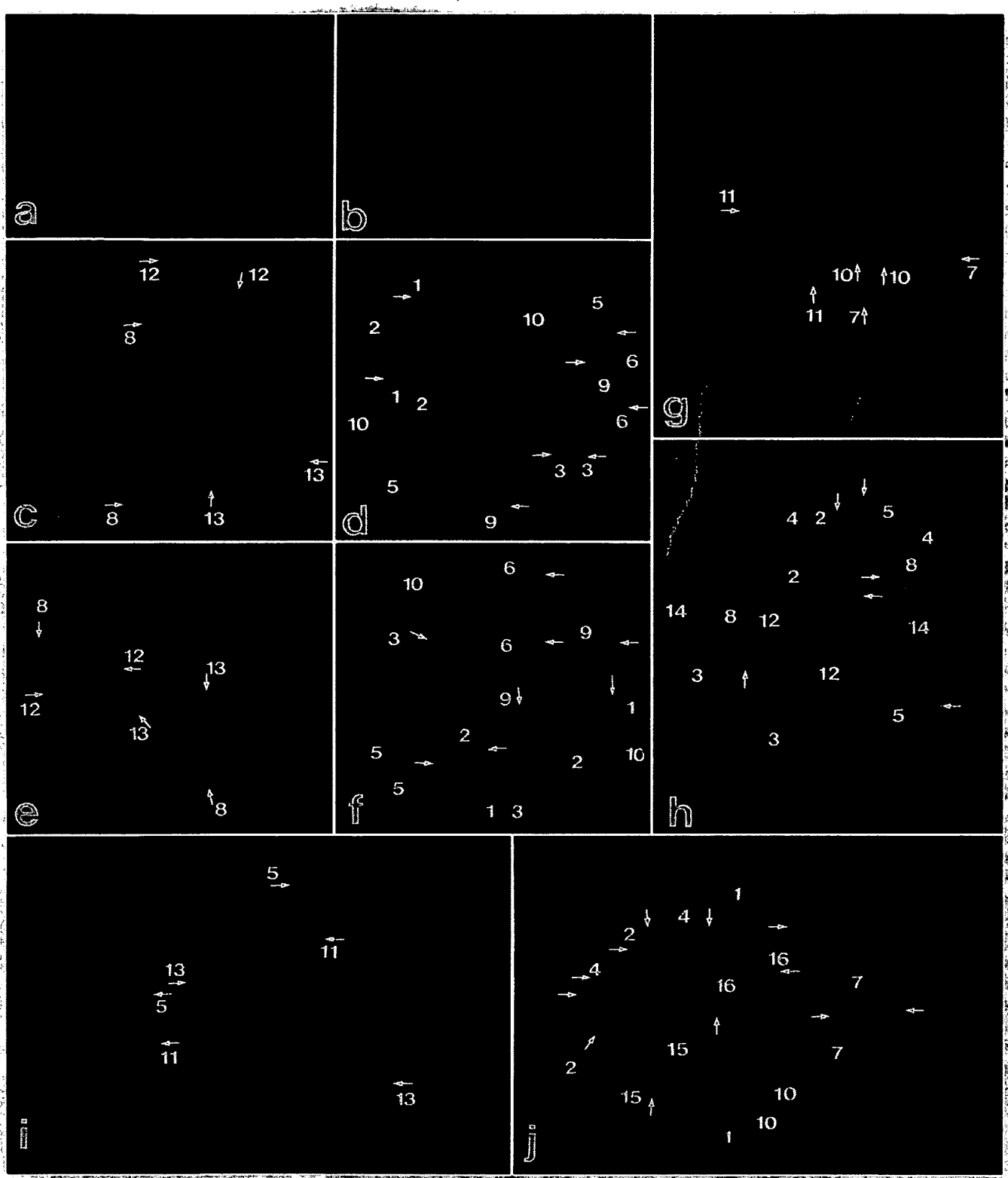


Fig. 2. Fluorescence in situ hybridization of probe pAm1 to chromosomes of *Avena* species. (*a*) Metaphase plate of *A. eriantha* showing hybridized chromosomes visualized in red. (*b*) The same cell as in *a* counterstained with DAPI. Arrows indicate the hybridization sites to A-genome chromosomes. (*c*) Metaphase plate of *A. murphyi* cc7120 showing hybridized chromosomes visualized in red. (*d*) The same cell as in *c* showing hybridization signals in pink on bluish chromosomes, obtained by double exposure using two different filters. Arrows indicate unhybridized segments of the C genome chromosome. (*e*) Metaphase plate of *A. murphyi* CAV 2832 showing hybridization sites in red. Arrows indicate hybridized segments of the A-genome chromosomes. (*f*) The same cell as in *e* showing hybridization signals in pink. Arrows indicate unhybridized segments of the C-genome chromosomes. (*g*) Metaphase plate of *A. maroccana* showing hybridization sites in red. Arrows indicate the hybridized segments of A-genome chromosomes. (*h*) The same cell as in *g* showing hybridization signals in pink. Arrows indicate unhybridized segments of the C-genome chromosomes. (*i*) Metaphase plate of *A. sativa* showing hybridization sites in red. Arrows indicate the hybridized segments of the A-genome chromosome. (*j*) The same cell as in *i* showing hybridized signals in pink. Arrows indicate the unhybridized segments of the C-genome chromosomes. In *d*, *f*, *h*, and *j*, the numbers indicate C-genome chromosomes. In *c*, *e*, *g*, and *i*, the numbers indicate A-genome chromosomes.

segments were identified as 2C, 5C, and 8C. Within the A genome, three blue chromosome pairs contained red segments distally located on their long arms. These chromosomes were identified as 7A, 10A, and 11A. No hybridization sites were identified in the remaining four chromosome pairs from the A genome.

FISH (Figs. 2*i*–2*j*) and enzymatic in situ hybridization (Fig. 3) with pAm1 revealed hybridization signals on 10 chromosome pairs in all metaphases of *A. sativa* cvs. Pandora and Prevision, *A. byzantina* cv. Kanota, and *A. sterilis*. Seven out of 10 chromosomes showed hybridization patterns similar to those described above for C-genome chromosomes. Within this set, five chromosome pairs, identified as 2C, 7C, 10C, 15C, and 16C, presented the hybridization signal distributed over the whole short arm with segments of variable length on the long arm. Moreover, chromosome 10C presented a strong signal in the middle of the unhybridized segment on the long arm. On one chromosome pair, identified as 4C, Am1 sequences were dispersed on the centromeric region and small segments of both arms, whereas the distal regions on both chromosome arms were unhybridized. The chromosome pair 1C was homogeneously hybridized, except for a small interstitial region on the long arm. Three chromosome pairs from the A/D genomes had a hybridization signal distally located on their long arms. These chromosomes were tentatively designated 5A, 11A, and 12A or 13A. The remaining 11 chromosome pairs were unhybridized.

Fig. 3. Metaphase plate of *A. sterilis* after in situ hybridization with biotinylated pAm1. Hybridization signals are visualized in black. Arrows indicate unhybridized segments of C-genome chromosomes. Arrowheads indicate hybridized segments of the A-genome chromosome. Numbers indicate C-genome chromosomes. A-genome chromosomes are indicated by numbers followed by the letter A.

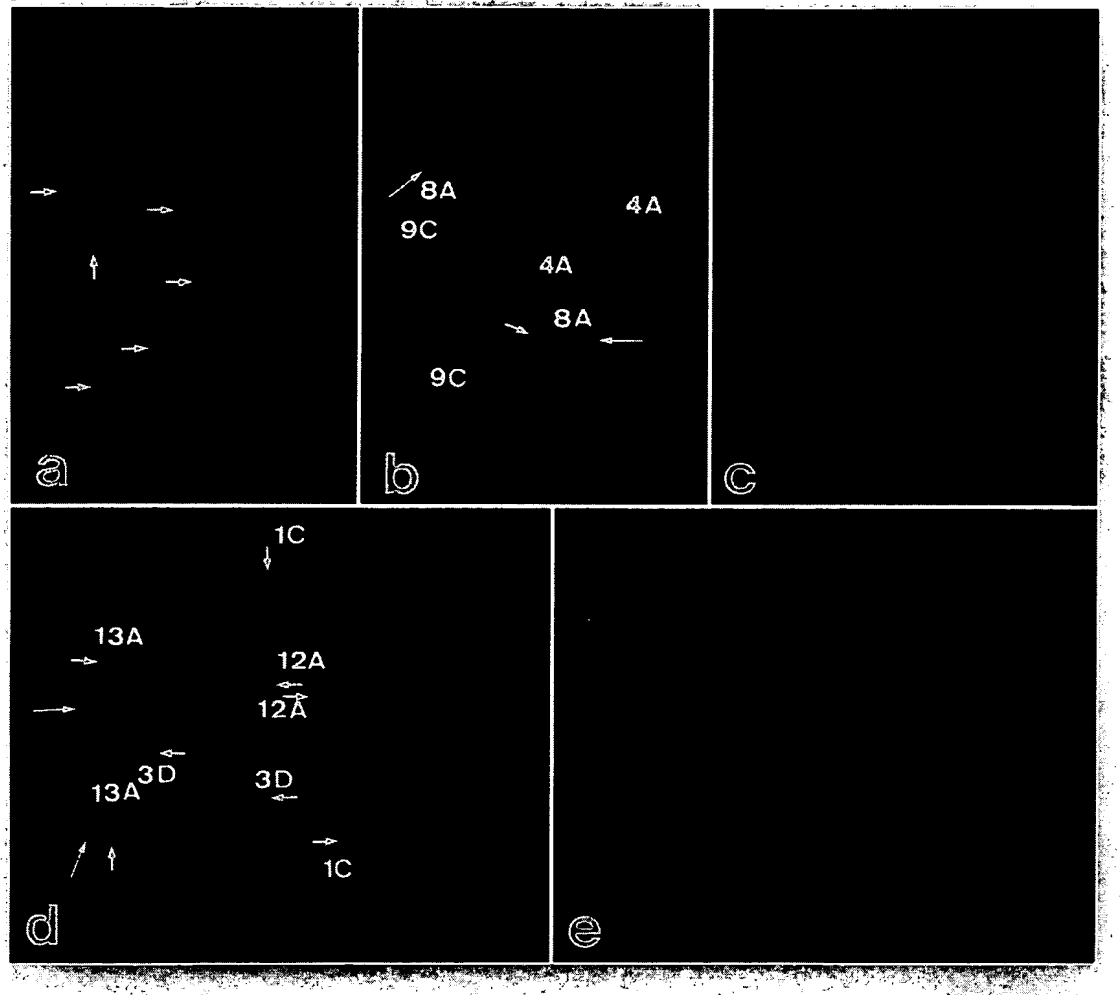
Localization of 18S, 25S, and 5.8S ribosomal genes (rDNA)

To further investigate the genome identity of the satellited (SAT) chromosomes in the tetraploid and hexaploid species, simultaneous in situ hybridizations using pTa71 for rDNA and pAm1 were performed (Fig. 4).

In situ hybridization to *A. murphyi* and *A. maroccana* with the digoxigenin-labeled probe pTa71, detected with fluorescein conjugated to anti-digoxigenin, revealed six sites on metaphase chromosomes (Fig. 4*a*). This indicates that three chromosome pairs in tetraploid oats carry ribosomal loci. Only two out of three pairs of labelled chromosomes showed the secondary constriction usually associated with SAT chromosomes. Simultaneous in situ hybridization with pTa71 (green in Fig. 4*b*) and pAm1 (orange in Fig. 4*b*) showed that the two pairs of chromosomes with NORs belonged to the A genome. One of them showed a hybridization signal in the short arm with pTa71 and a hybridization signal in the long arm with pAm1. NOR-bearing chromosomes were identified by their satellite morphology and arm ratio in *A. murphyi* (4A and 8A) and *A. maroccana* (1A and 10A), following the karyotype reported by Fominaya et al. (1988*b*). Moreover, the chromosomes carrying Am1 sequences were identified as 8A and 10A of *A. murphyi* and *A. maroccana*, respectively. The third pair showing NOR sequences belonged to the C genome and was identified as 9C and 8C in *A. murphyi* and *A. maroccana*, respectively.

When FISH analysis was performed on metaphase spreads of hexaploid species with the pTa71 clone, eight hybridization sites were detected: six brightly fluorescing major sites and two additional weakly fluorescing minor sites of rDNA (Fig. 4*d*). In all cases, the loci for the NOR sequences appeared in the short arms of the chromosomes.

Fig. 4. In situ hybridization of metaphase plates of *A. murphyi* (*a*, *b*, *c*) and *A. sterilis* (*d*, *e*). (*a*) Digoxigenin-labeled pTa71 probe detected with conjugated fluorescein (green). Arrows show rDNA hybridization sites. (*b*) Simultaneous visualization of hybridization sites to pTa71 (green) and pAm1 (orange). Arrows show the chromosome pair carrying hybridization with pTa71 (short arm) and pAm1 (long arm). (*c*) The same cell as in *a* and *b* counterstained with DAPI. (*d*) A double exposure to show the positions of the hybridization sites of pTa71 (green) and pAm1 (orange). Arrows indicate rDNA sites and the chromosome pair that hybridizes with rDNA and pAm1. (*e*) The same cell as in *d* counterstained with DAPI.



The genome identity of the NOR chromosomes was further revealed by double labelling with the probes pTa71 (green in Fig. 4*d*) and pAm1 (orange in Fig. 4*d*). Two pairs of chromosomes showed a NOR-hybridization site at the interstitial region of the short arms and no hybridization sites for the pAm1 probe. This reveals that these chromosomes belong to either the A or the D genome. One chromosome pair had a NOR in the short arm and a hybridization signal with pAm1 in the long arm. This chromosome pair closely resembled the corresponding SAT chromosome pairs identified as 8A or 10A in the tetraploids *A. murphyi* and *A. maroccana*, respectively. In the hexaploids this chromosome pair was identified as 13A. Consequently, the other two SAT chromosome pairs were identified as 12A and 3D by comparing them with the C-banded karyotype of the hexaploid *A. byzantina* (Linares et al. 1992). One chromosome pair showed hybridization with the pAm1 probe over its length and also presented a small ribosomal site on the short arm. This was identified as 1C by morphological comparison with the *A. byzantina* karyotype.

Discussion

Solano et al. (1992) suggested that Am1 was a satellite DNA sequence on the basis of its organization, namely long tandem arrays of short repeating units. Results described in this paper confirm this hypothesis: (*i*) its abundance in the genomes of species with the C genome indicates that Am1 is highly repeated in that genome; (*ii*) its genome-specific nature means that it is absent in species with closely related genomes, agreeing with the widely accepted assumption that satellite DNA diverges rapidly (Singer 1982); and (*iii*) the length and structure of the monomer correspond to other plant satellite DNAs. Many

of the plant satellite DNA sequences described so far are composed either of units of about 60 bp (Kato et al. 1984) or monomers longer than 60 bp but with subrepeats of this length (Grellet et al. 1986; Xia et al. 1993; Ingham et al. 1993). Inverted repeats, like those found in Am1, have also been observed in repeated DNA from *Secale cereale* (Bedbrook et al. 1980), *Vicia faba* (Kato et al. 1984), and *Brassica napus* (Xia et al. 1993), although their significance is not known. These observations indicate that Am1 satellite DNA evolves following patterns common to other plant satellite DNAs, i.e., duplications of shorter units followed by massive amplifications of these new units, units that are as large as 51 bp in this oat satellite DNA. The high-levels of hybridization found between the probe pAm1 and the chromosomes of the oat species with the C genome indicates that the amplification of Am1 has occurred recently in the evolutionary history of oats.

Satellite DNA is usually located in regions of constitutive heterochromatin that are often found close to the telomeres and centromeres of plant chromosomes. The satellite DNA clusters in rye (McIntyre et al. 1990; Cuadrado and Jouve 1994) and *Agropyron* (Xin and Appels 1988) were found mostly near the telomeres, while in hexaploid wheat (Hutchinson and Lonsdale 1982), barley (Dennis et al. 1980), and *Brassica nappus* (Xia et al. 1993) they were primarily localized around the centromeres. On the other hand, a fraction of satellite DNA from *Anemona blanda* (Hagemann et al. 1993) was located in all the prominent intercalary C bands, and a satellite DNA family was specifically located on the intercalary proximal bands of *Crepis capillaris* (Jamilena et al. 1993). In oats, a high degree of correlation can be seen between the distribution and intensity of the hybridization signals of pAm1 on the C-genome chromosomes and their previously described corresponding C-banding patterns (Fominaya et al. 1988*a*, 1988*b*; Linares et al. 1992; Jellen et al. 1993*a*, 1993*b*). Although it seems improbable that only one satellite DNA exists in the oat C genome, it may be inferred that the stained heterochromatin blocks of the C-genome chromosomes are composed of tandem arrays of the Am1 satellite.

Since pAm1 only hybridized with 14 chromosomes in hexaploid oats, it may be deduced that the unknown donor species of the D genome is not related to the diploid C genome species. Analysis of the distribution of the Am1 satellite on the C-genome chromosomes revealed differences depending on the species studied. Chromosomes from *A. eriantha* and *A. clauda* showed an even distribution of the Am1 satellite along the entire length of their 14 chromosomes (Fig. 2). In the tetraploid and hexaploid species, several of the 14 C genome chromosomes had terminally located nonhybridizing regions (Figs. 2 and 3). A possible explanation of these observations would be based on the existence of intergenomic translocations between chromosomes belonging to the A/D genome and the C genome. This was suggested by Fominaya et al. (1988*b*) using data from C-banding observations and by Rajhathy and Thomas (1974) from the analysis of meiotic chromosome pairing of interspecific hybrids. Recently these proposals were confirmed in several papers arising from research using genomic in situ hybridization (Leggett et al. 1994; Chen and Armstrong 1994; Jellen et al. 1994*b*).

Leggett et al. (1994) reported the existence of A–C intergenomic translocations in the tetraploid *A. maroccana*. Jellen et al. (1994*b*) reported the same for the tetraploid species *A. maroccana* and *A. murphyi*. These authors detected C chromatin on four A chromosome pairs that included two pairs of SAT chromosomes in both tetraploid species and A chromatin on one C chromosome pair in *A. maroccana* and on two pairs in *A. murphyi*. In contrast, these results showed the presence of Am1 sequences on three A chromosome pairs, including only one pair of SAT chromosomes. Further, there was an absence of Am1 sequences on three C chromosome pairs in *A. maroccana* and on four pairs in *A. murphyi*.

Chen and Armstrong (1994) and Jellen et al. (1994*a*, 1994*b*) also reported A/D–C intergenomic translocations in hexaploid species. These authors detected C chromatin on six A chromosome pairs, including two pairs of SAT chromosomes, and A chromatin on three C chromosome pairs. In contrast, the presence of Am1 sequences on three A chromosome pairs and the absence of Am1 sequences on seven C chromosome pairs were observed in this study.

These discrepancies could easily be explained by the distinct nature of the DNA probe used in each experiment. Whereas whole intergenomic translocations are detected when genomic DNA from diploid species is used as probe, only those intergenomic translocations involving Am1 sequences are detected in this study. If Am1 is a satellite sequence distributed throughout the C-genome heterochromatin, a correlation between the presence of Am1 sequences and the presence of heterochromatin in the A chromosomes can be established. It was found that the three A/D–C translocated chromosomes bearing Am1 sequences also presented patent C-bands. These were tentatively identified as chromosomes 5A, 11A, and 13A, as described by Linares et al. (1992). Jellen et al. (1994*b*) have recently suggested that a substantial portion of the C-genome chromatin is euchromatic in the polyploid species. These results agree with this hypothesis, since in situ hybridization with pAm1 did not detect several of the described A/D–C translocations. Moreover, terminal regions of C-genome chromosomes that failed to hybridize with pAm1 were not detected as C–A/D translocations after genomic in situ hybridization.

The existence of deletions involving repeated sequences, which seems to accompany species evolution (Flavell 1982), could explain the differences observed in the distribution of Am1 sequences between C-diploid and polyploid species.

NORs contain expressed 18S–5.8S and 26S ribosomal genes that are cytologically identifiable as a secondary constriction on satellite chromosomes. Rajhathy and Thomas (1974) and Fominaya et al. (1988*b*) reported two pairs of SAT chromosomes in tetraploid oats using cytological and silver staining techniques, respectively. Linares et al. (1992) and Jellen et al. (1993*a*, 1993*b*) reported three pairs of SAT chromosomes in hexaploid oats using silver staining and C-banding analysis, respectively. Jellen et al. (1994*a*), using Southern and radioactive in situ hybridization analyses, also reported the presence of three pairs of SAT chromosomes in the common cultivated oat. In contrast, these results identified a number of rDNA sites in excess of the number of expected NORs. Three pairs of chromosomes

with rDNA sites, two with major and one with minor sites, were detected in tetraploid oats. Four pairs of chromosomes with rDNA sites, three with major and one with minor sites, were detected in hexaploid oats. These discrepancies could easily be explained by the sensitivity of the technique used. Although the major rDNA sites corresponding to SAT chromosomes can be detected by the presence of the secondary constriction, silver staining, or radioactive in situ hybridization, both major and minor rDNA sites are detected by FISH.

Ribosomal and pAm1 probes were used in simultaneous FISH to differentiate the chromosomes that carry rDNA genes. In tetraploid oats, the two pairs with major rDNA sites belong to the A genome. The pair with minor rDNA sites belongs to the C genome. Similarly, in hexaploid oats, the three pairs with major rDNA sites belong to the A/D genomes and the pair with the minor rDNA site belongs to the C genome. Based on Southern analysis of different aneuploid lines of hexaploid oat, Jellen et al. (1994*a*) suggested that one of the SAT chromosomes belonging to the A genome could bear NOR sequences translocated from a C-genome chromosome. These results do not support this hypothesis, since only one SAT chromosome had Am1 satellite sequences and these were distally located on the long arm. This chromosome seems to be homologous to the one described in the tetraploid species (Figs. 4*b* and 4*d*) and consequently would belong to the A genome.

In conclusion, the physical locating of ribosomal and repetitive DNA sequences in the chromosomes of tetraploid and hexaploid oats demonstrates the utility of in situ hybridization for (*i*) identifying the genome origin in these allopolyploid species, (*ii*) detecting the presence of intergenomic translocations, and (*iii*) unambiguously assigning the SAT chromosomes to specific genomes. The isolation of new repetitive sequences will allow a more complete picture of the genomic relationships in this genus to be obtained.

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References

Bedbrook, J.R., Jones, J., O'Dell, M., Thompson, A.D., and Flavell, R.B. 1980. A molecular description of telomeric heterochromatin in *Secale* species. Cell, **19**: 545–560.

Bennett, M.D., and Smith, J.B. 1976. Nuclear amounts in angiosperms. Proc. R. Soc. London B Biol. Sci. **274**: 227–274.

Chen, Q., and Armstrong, K. 1994. Genomic in situ hybridization in *Avena sativa*. Genome, **37**: 607–612.

Cuadrado, A., and Jouve, N. 1994. Mapping and organization of highly-repeated DNA sequences by means of simultaneous and sequential FISH and C-banding in 6*x*-triticale. Chromosome Res. **2**: 331–338.

Dennis, E.S., Gerlach, W.L., and Peackock, W.J. 1980. Identical polypyrimidine–polypurine satellite DNAs in wheat and barley. Heredity, **44**: 349–366.

Fabijanski, S., Fedak, G., Armstrong, K., and Altosaar, I. 1990. A repeated sequence probe for the C genome in *Avena* (oats). Theor. Appl. Genet. **79**: 1–7.

Flavell, R.B. 1982. Sequence amplification, deletion and rearrangement: major sources of variation during species divergence. *In* Genome evolution. *Edited by* G.A. Dover and R.B. Flavell. Academic Press, London. pp. 301–323.

Fominaya, A., Vega, C., and Ferrer, E. 1988*a*. Giemsa C-banded karyotypes of *Avena* species. Genome, **30**: 627–632.

Fominaya, A., Vega, C., and Ferrer, E. 1988*b*. C-banding and nucleolar activity of tetraploid *Avena* species. Genome, **30**: 633–638.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Grellet, F., Delcasso, D., Panabieres, F., and Delseny, M. 1986. Organization and evolution of a higher plant alphoid-like satellite DNA sequence. J. Mol. Biol. **187**: 495–507.

Gupta, P.K., Giband, M., and Altosaar, I. 1992. Two molecular probes characterizing the A and C genomes in the genus *Avena* (oats). Genome, **35**: 916–920.

Hagemann, S., Scheer, B., and Schweizer, D. 1993. Repetitive sequences in the genome of *Anemona blanda*: identification of tandem arrays and of dispersed repeats. Chromosoma, **102**: 312–324.

Heslop-Harrison, J.S., Schwarzacher, T., Anamthawat-Jonsson, K., Leitch, A.R., Shi, M., and Leitch, I.J. 1991. In situ hybridization with automated chromosome denaturation. J. Methods Cell Mol. Biol. **3**: 109–116.

Hoisington, D.A. 1992. Laboratory protocols. International Maize and Wheat Improvement Center (CIMMYT) Applied Molecular Genetics Laboratory, Mexico.

Hutchinson, J., and Lonsdale, D.M. 1982. The chromosomal distribution of cloned highly repetitive sequences from hexaploid wheat. Heredity, **48**: 371–376.

Ingham, L.D., Hanna, W.W., Baier, J.W., and Hannah, L.C. 1993. Origin of the main class of repetitive DNA within selected *Pennisetum* species. Mol. Gen. Genet. **238**: 350–356.

Jamilena, M., Ruiz Rejón, C., and Ruiz Rejón, M. 1993. Repetitive DNA sequence families in *Crepis capillaris*. Chromosoma, **102**: 272–278.

Jellen, E.N., Rooney, W.L., Phillips, R.L., and Rines, H.W. 1993*a*. Characterization of the hexaploid oat *Avena byzantina* cv. Kanota monosomic series using C-banding and RFLPs. Genome, **36**: 962–970.

Jellen, E.N., Phillips, R.L., and Rines, H.W. 1993*b*. C-banded karyotypes and polymorphisms in hexaploid oat accessions (*Avena* spp.) using Wright's stain. Genome, **36**: 1129–1137.

Jellen, E.N., Phillips, R.L., and Rines, H.W. 1994*a*. Chromosomal localization and polymorphisms of ribosomal DNA in oat (*Avena* spp.). Genome, **37**: 23–32.

Jellen, E.N., Gill, B.S., and Cox, T.S. 1994*b*. Genomic in situ hybridization differentiates between A/D- and C-genome chromatin and detects intergenomic translocations in polyploid oat species (genus *Avena*). Genome, **37**: 613–618.

John, B., and Miklos, G.L.G. 1979. Functional aspects of satellite DNA and heterochromatin. Int. Rev. Cytol. **58**: 1–14.

Kato, A., Yakura, K., and Tanifuji, S. 1984. Sequence analysis of *Vicia faba* repeated DNA, the FokI repeat element. Nucleic Acids Res. **12**: 6415–6426.

Kenton, A., Parokonny, A.S., Gleba, Y.Y., Bennett, M.D. 1993. Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. Mol. Gen. Genet. **240**: 159–169.

Leggett, J.M. 1992. Classification and speciation in *Avena*. *In* Oat science and technology. *Edited by* H.G. Marshall and M.E. Sorrells. Agronomy Monograph 33. American Society of Agronomy (ASA) and Crop Science Society of America (CSSA) Madison, Wis., U.S.A. pp. 29–52.

Leggett, J.M., Thomas, H.M., Meredith, M.R., Humphreys, M.W.,

Morgan, W.G., Thomas, H., and King, I.P. 1994. Intergenomic translocations and the genomic composition of *Avena maroccana* Gdgr revealed by FISH. Chromosome Res. **2**: 163–164.

Linares, C., Vega, C., Ferrer, E., and Fominaya, A. 1992. Identification of C-banded chromosomes in meiosis and the analysis of nucleolar activity in *Avena byzantina* C Koch cv 'Kanota'. Theor. Appl. Genet. **83**: 650–654.

Maluszynska, J., and Heslop-Harrison, J.S. 1993. Physical mapping of rDNA loci in *Brassica* species. Genome, **36**: 774–781.

McIntyre, C.L., Clarke, B.C., and Appels, R. 1988. Amplification and dispersion of repeated DNA sequences in the *Triticeae*. Plant. Syst. Evol. **160**: 39–59.

McIntyre, C.L., Pereira, S., Moran, L.B., and Appels, R. 1990. New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. Genome, **33**: 635–640.

Morikawa, T. 1985. Identification of the 21 monosomic lines in *Avena byzantina* C Koch cv 'Kanota'. Theor. Appl. Genet. **70**: 271–278.

Rajhathy, T., and Thomas, H. 1974. Cytogenetics of oats (*Avena* L). Misc. Publ. Genet. Soc. Can. No. 2. pp. 1–90.

Sánchez de la Hoz, P., and Fominaya, A. 1989. Studies of isozymes in oat species. Theor. Appl. Genet. **77**: 735–741.

Singer, M.F. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. **76**: 67–112.

Solano, R., Hueros, G., Fominaya, A., and Ferrer, E. 1992. Organization of repeated sequences in species of the genus *Avena*. Theor. Appl. Genet. **83**: 602–607.

Tsujimoto, H., and Gill, B.S. 1991. Repetitive DNA sequences from polyploid *Elymus trachycaulus* and the diploid progenitor species: detection and genomic affinity of *Elymus* chromatin added to wheat. Genome, **34**: 782–789.

Walbot, V., and Cullis, C.A. 1985. Rapid genomic change in higher plants. Annu. Rev. Plant. Physiol. **36**: 367–399.

Xia, X., Selvaraj, G., and Bertrand, H. 1993. Structure and evolution of a highly repetitive DNA sequence from *Brassica napus*. Plant. Mol. Biol. **21**: 213–224.

Xin, Z.Y., and Appels, R. 1988. Occurrence of rye (*Secale cereale*) 350-family DNA sequences in *Agropyron* and other Triticeae. Plant. Syst. Evol. **160**: 65–76.

Zhao, X., Xu, T., Xie, Y., and Wu, R. 1989. Genome-specific repetitive sequences in the genus *Oryza*. Theor. Appl. Genet. **78**: 201–209.

Physical mapping of rDNA loci in *Brassica* species

J. MALUSZYNSKA
Department of Plant Anatomy and Cytology, Silesian University, Jagiellonska 28, 40-032 Katowice, Poland
AND
J.S. HESLOP-HARRISON[1]
Karyobiology Group, Department of Cell Biology, John Innes Centre for Plant Science Research, Colney Lane, Norwich, U.K. NR4 7UJ

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The number of major rDNA loci (the genes coding for 18S–5.8S–26S rRNA) was investigated in the economically important *Brassica* species and their wild relatives by in situ hybridization of an rDNA probe to metaphase chromosomes and interphase nuclei. The diploid species *B. nigra* (B genome) has two major pairs of rDNA loci, *B. oleracea* (C genome) has two major pairs and one minor pair of loci, while *B. campestris* (A genome) has five pairs of loci. Among the three tetraploid species arising from these three diploid ancestors, *B. carinata* (BBCC genomes) has four loci, *B. juncea* (AABB genomes) has five major pairs and one minor pair of loci, and *B. napus* (AACC genomes) has six pairs of loci, indicating that the number of loci has been reduced during evolution. The complexity of the known rDNA restriction fragment length polymorphism patterns gave little indication of number of rDNA loci. It is probable that chromosome rearrangements have occurred during evolution of the amphidiploid species. The data will be useful for physical mapping of genes relative to rDNA loci, micro- and macro-evolutionary studies and analysis of aneuploids including addition and substitution lines used in *Brassica* breeding programs.

Key words: *Brassica*, centromeric DNA, genetic maps, nuclear architecture, ribosomal DNA, evolution, Brassicaceae, Cruciferae, gene mapping.

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Des espèces de *Brassica* d'importance économique et des espèces indigènes apparentées ont fait l'objet d'une étude, par hybridation in situ d'une sonde d'ADNr sur des chromosomes en métaphase et des noyaux en interphase, pour en déterminer les nombres de locus majeurs d'ADNr (gènes codant les ARNr 18S–5.8S–26S). Chez les espèces diploïdes, le *B. nigra* (génome B) a deux paires majeures de locus d'ADNr, le *B. oleracea* (génome C) possède deux paires majeures et une paire mineure de locus et, le *B. campestris* (génome A) présente cinq paires de locus. Des trois espèces tétraploïdes dérivées de ces ancêtres diploïdes, le *B. carinata* (génomes BBCC) a quatre locus, le *B. juncea* (génomes AABB) possède cinq paires majeures et une paire mineure de locus et, le *B. napus* (génomes AACC) présente six paires de locus, ce qui indique que le nombre de locus a été réduit au cours de l'évolution. La complexité des profils de polymorphisme des longueurs de fragments de restriction fournit peu d'indication sur le nombre de locus des ADNr. Il est probable que des réarrangements chromosomiques sont survenus au cours de l'évolution des espèces amphidiploïdes. Ces données seront utiles pour la cartographie physique des gènes ayant trait aux locus d'ADNr, aux études de micro- et de macro-évolution et à l'analyse des aneuploïdes, incluant les lignées d'addition et de substitution utilisées dans les programmes d'amélioration des *Brassica*.

Mots clés : *Brassica*, ADN centromérique, carte génétique, architecture nucléaire, ADN ribosomal, évolution, Brassicaceae, Cruciferae, cartographie des gènes.

[Traduit par la rédaction]

Introduction

In plants, 18S–5.8S–26S ribosomal RNA genes (rRNA genes or rDNA) are present as many hundreds of tandemly repeated units of the three genes and intergenic spacers, at one or more pairs of loci within the genome. The karyotypes of the various species of *Brassica* have been studied for many years (see Prakash and Chopra 1991 for a review). When expressed, the rRNA genes produce a nucleolus, and secondary constrictions, nucleolar organizer regions (NORs), are visible at some loci on metaphase chromosomes. Olin-Fatih and Heneen (1992) presented C-banded karyotypes of three *Brassica* species. In late prophase preparations, they were able to identify one chromosome pair with an NOR in *B. campestris*, *B. oleracea*, and their amphidiploid, *B. napus*. Other NORs may have been present but would not be visible as secondary constrictions in the preparations because they showed little or no expression

Delseny et al. (1990) described the organization and polymorphism of rRNA genes in diploid and amphidiploid *Brassica* species using restriction fragment length polymorphism (RFLP) analysis. They discussed the need for analysis of the number of loci of rDNA within the genomes to complement the polymorphism studies. Analysis of NOR distribution and organization in rDNA-carrying chromosomes is important to understand the events that have happened during divergence and hybridization of the species.

[1]Author for correspondence.

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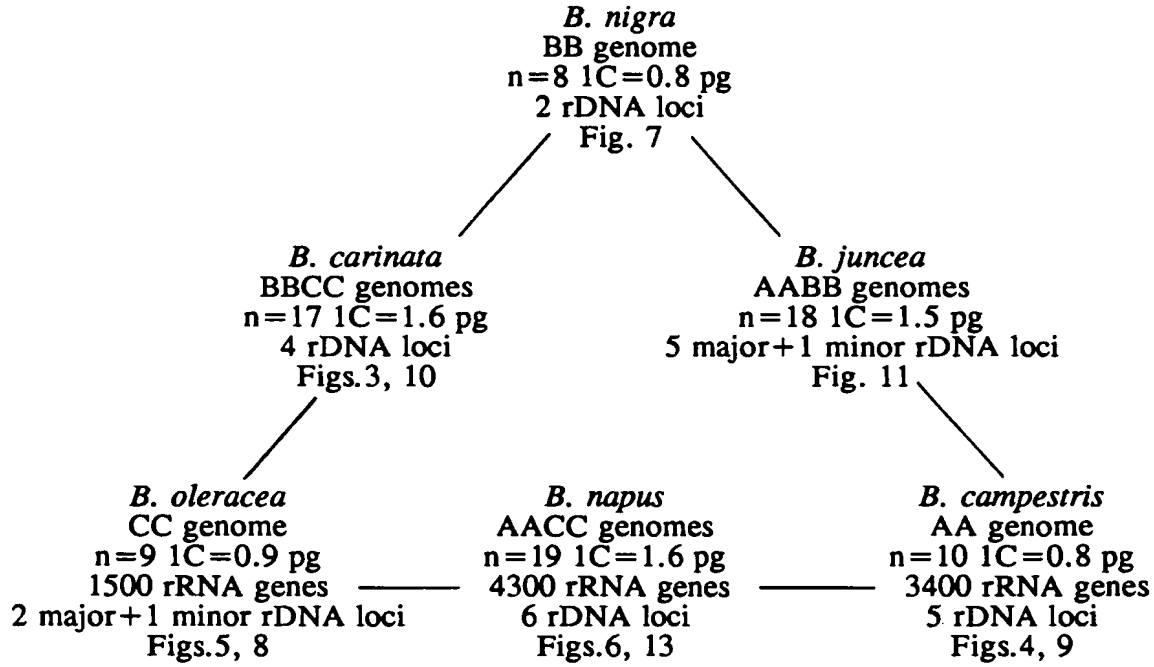


FIG. 1. The relationships (after U 1935), chromosome numbers, genome designations, DNA contents (Bennett and Smith 1976), and the number of rDNA loci (Bennett and Smith 1991) for three diploid and three amphidiploid *Brassica* species (see Figs. 3–13).

Within the tribe Triticeae, phylogenies have been widely studied by both molecular cytogenetic and molecular biological analyses, particularly using the rRNA genes. Together, the methods have provided valuable and complementary data about rDNA sequence evolution and variation. The copy number of the repeat unit at single loci, and number of rDNA loci within the genome, are extremely variable between related species. The number of rDNA loci in diploid species ($2n = 2x = 14$) varies from one pair in rye (*Secale cereale* L.) to five pairs in barley (*Hordeum vulgare* L.; Leitch and Heslop-Harrison 1992). In hexaploid wheat ($2n = 6x = 42$; *T. aestivum* L. *emend* Thell.), a species that arose from diploid and tetraploid progenitors some 10 000 years ago, the rDNA lies on five pairs of chromosomes (Mukai et al. 1991). However, the number of copies has been greatly reduced at three loci from two of the ancestral genomes, while the two loci from the third genome donor both remained large. Changes in rDNA-repeat copy number at single loci can be rapid: when wheat, or other species, are maintained in tissue culture, the number of loci and rDNA-repeat copy number can change substantially within a few years (Leitch et al. 1993).

The ability to look at rDNA copy number and site distribution is important for examining relationships and evolutionary changes between genomes and for classifying chromosome types within genomes. Fluorescent in situ hybridization to chromosome spread preparations using labelled DNA probes is the method of choice for mapping repetitive genes such as those for rRNA (Heslop-Harrison 1991), because it is not affected by the presence of minor variants within major loci nor extreme differences in copy number between loci. In conjunction with fluorochrome staining, in situ hybridization is an extremely valuable method for investigating chromosome morphology, particularly in species with small chromosomes (see Maluszynska and Heslop-Harrison 1991).

In the present work, we examined the number of rDNA loci, their locations along chromosomes, aspects of their decondensation at interphase, and approximate relative copy numbers at different loci in major crop species of *Brassica* and their diploid and tetraploid ancestors or relatives.

Materials and methods

The species used were *B. campestris* L. turnip John Innes accession OB441 (synonym *B. rapa* L.), *B. nigra* (L.) Koch black mustard OB693, *B. oleracea* L. Savoy cabbage Sabanda OB420, *B. carinata* A. Braun. Abyssinian mustard National Vegetable Research Station accession NVRS08.006807, *B. napus* L. winter oil seed rape Falcon, and *B. juncea* (L.) Czrm. brown mustard J/551/7/9. Seeds were germinated on MS medium (Murashige and Skoog 1962) with 0.8% agar, or moist filter paper, for 3–4 days. Ploidy levels, chromosome numbers, and genome designations are given in Fig. 1. Seedlings were fixed in 100% methanol – glacial acetic acid (3:1) after 3–4 h treatment with 2 mM 8-hydroxyquinoline to accumulate divisions and stored at −20°C until use.

Chromosome preparation

Chromosome preparation followed techniques described for *Arabidopsis* species (Maluszynska and Heslop-Harrison 1993) modified after Schwarzacher et al. (1980). Briefly, fixed seedlings were washed in 0.01 M citric acid – sodium citrate (pH 4.8) for 15 min and digested in 2% (w/v) cellulase (Calbiochem) in 20% (v/v) pectinase (from *Aspergillus niger*, Sigma) for 1 h at 37°C. A few (typically five to six) root tips per slide were squashed in a drop of 45% acetic acid. The cover slip was removed after freezing on dry ice and the preparation was air dried. The quality of the chromosome spread was checked after staining with 2 $\mu g \cdot mL^{-1}$ 4′-6-

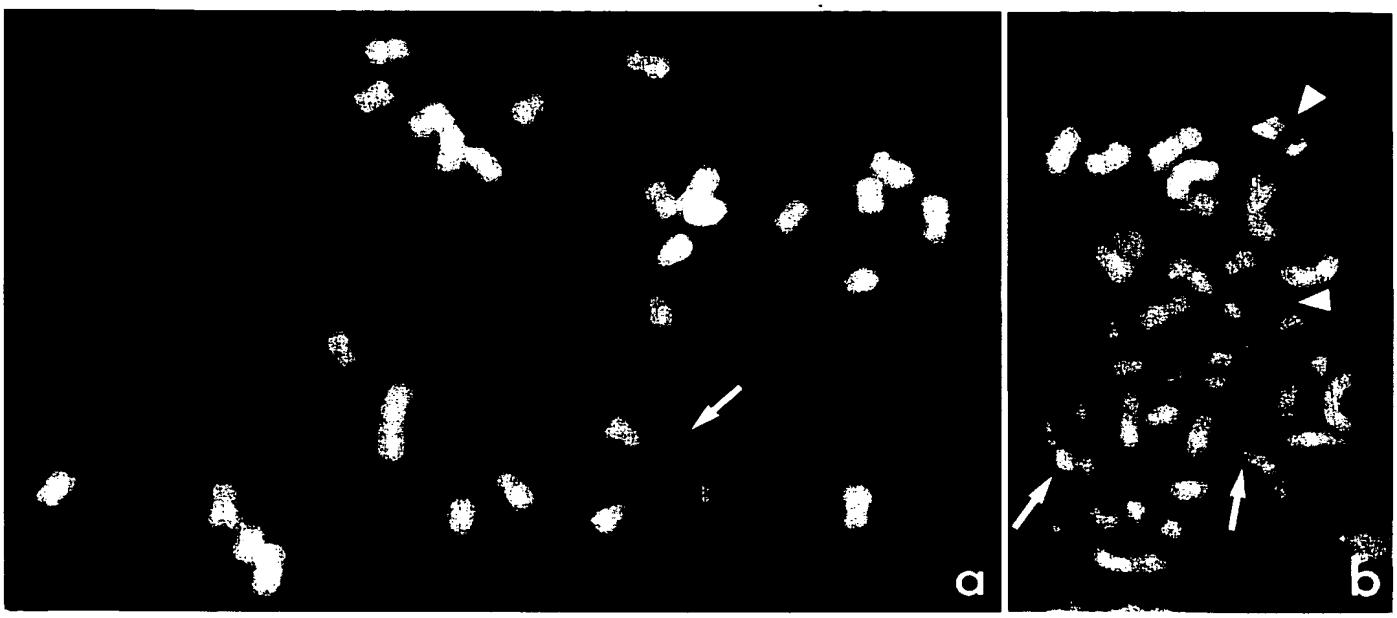


FIG. 2. DAPI-stained metaphases of (*a*) *Brassica carinata* and (*b*) *Brassica napus*. (*a*) Arrow indicates threads of weakly DAPI-stained DNA presumably associated with nucleolar organizing chromosomes that were active at the previous interphase. (*b*) Arrowheads indicate some chromosome regions that contain GC-rich sequences that are weakly stained with the AT-enhanced fluorochrome DAPI. Arrows indicate some chromosome regions that become DAPI-positive C-bands after hybridization (as shown in the same metaphase after hybridization in Fig. 6*a*).

diamidino-2-phenylindole (DAPI) for 15 min in McIlvaine's buffer (18% 0.1 M citric acid, 82% Na_2HPO_4 adjusted to pH 7), and chromosome preparations were photographed for morphological analysis (Fig. 2). Satisfactory preparations were destained with methanol – acetic acid (3:1) for 3–6 h at 4°C, rinsed with three changes of 100% methanol, and air dried. The slides were stored at room temperature for several days before in situ hybridization.

In situ hybridization

In situ hybridization was carried out using digoxigenin-labelled DNA probes. Sites of hybridization were detected with anti-digoxigenin antibody conjugated to fluorescein isothiocyanate (FITC; Heslop-Harrison et al. 1991).

Clone pTa71 was used as a probe for in situ hybridization. It contains a 9-kb *Eco*RI repeat unit of ribosomal DNA (rDNA) isolated from *T. aestivum* (Gerlach and Bedbrook 1979) and has been shown to be highly homologous to the *Brassica* rDNA sequences by Southern hybridization (Delseny et al. 1990). For use as a probe, DNA was labelled with digoxigenin-11-dUTP (Boeringher-Mannheim) by nick translation using standard techniques.

For in situ hybridization, DNA probes were mixed to a final concentration of 50 ng·mL^{-1} in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulphate), and 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate). Slides were treated with 100 μg·mL^{-1} DNase-free RNase in 2× SSC for 1 h at 37°C, washed twice in 2× SSC for 10 min at room temperature, dehydrated in a graded ethanol series and air dried.

The hybridization mixture (20 μL/slide) was added to the chromosome preparation and covered with a plastic cover slip. The chromosomes and DNA probe were denatured together in a humid chamber at 90°C for 7 min. Hybridization was carried out at 37°C in the chamber for 12–16 h. The humid chamber was a 40 mm high metal box with a close-fitting, sloping lid; eight slides were balanced on two glass rods lying inside on wet tissue. The box was floated in a water bath and the temperature was adjusted based on readings from a digital thermometer probe inside the box. After hybridization, cover slips were removed in 2× SSC at 42°C and then given a stringent wash for 10 min each in 50% (v/v) formamide in 2× SSC at 42°C, 2× SSC at 42°C, and 2× SSC at room temperature. The wash removes weakly hybridized sequences with less than 85% homology.

Hybridization sites were detected by transferring the slides to 4× SSC–Tween buffer (4× SSC, 0.2% (v/v) Tween 20) for 5 min, treating with 5% (w/v) bovine serum albumin in 4× SSC–Tween for 5 min, and then incubating with 20 μg·mL^{-1} anti-digoxigenin antibody (raised in sheep) conjugated with FITC for 1 h at 37°C. The slides were washed in 4× SSC–Tween at 37°C for 3 × 8 min.

For signal amplification, slides were blocked for 5 min with 5% (v/v) normal goat serum in 4× SSC–Tween and then incubated in 10 μg·mL^{-1} rabbit-anti-sheep FITC in 5% normal goat serum for 1 h at 37°C. The slides were washed in 4× SSC–Tween 3 × 8 min at 37°C.

Preparations were counterstained with DAPI (2 μg·mL^{-1}) and some slides were also stained with propidium iodide (1 μg·mL^{-1}). The slides were mounted in antifade solution (AF1, Citifluor). Slides were examined with a Zeiss epifluorescence microscope with filter set 02 and 09. Photographs were taken on Fujicolor Super HG 400 colour print film. Exposure times and printing of all micrographs of one subject type (DAPI stained or in situ signal) were similar.

Results

DAPI staining

DAPI staining showed the morphology of metaphase chromosomes by fluorescence microscopy (Figs. 2, 3*a*, 4*a*, 5*a*, and 6*a*). Staining of most chromosomes was less strong at the centromere and some chromosomes had regions with less staining (e.g., Figs. 2*c* and 3*a*). Some chromosomes had large, terminal regions that were

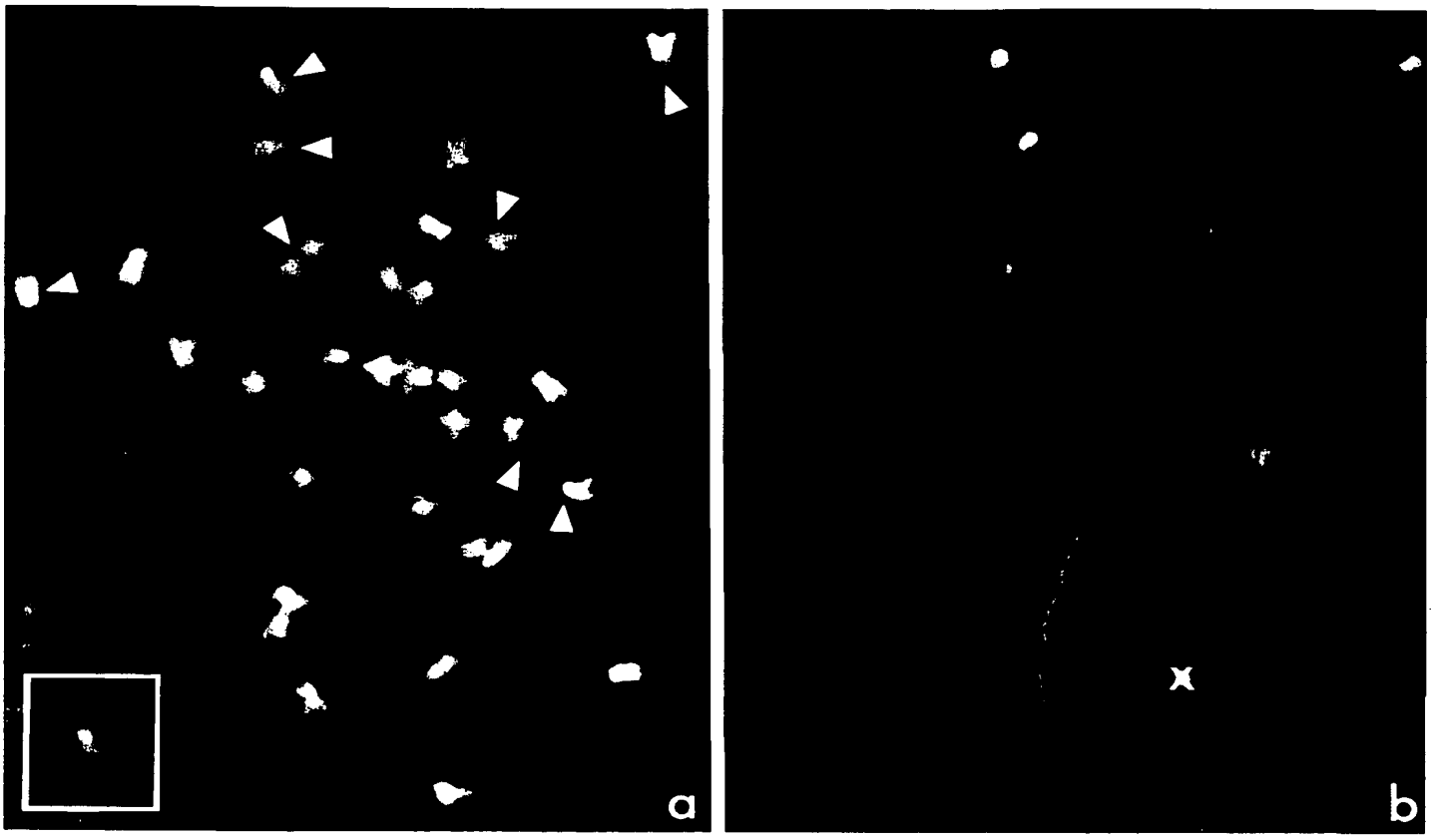


FIG. 3. *Brassica carinata* root-tip metaphase. (*a*) DAPI-stained chromosomes; arrowheads indicate those carrying rDNA hybridization sites. Inset is a chromosome separated slightly from the rest of the plate. (*b*) In situ hybridization showing four major pairs of rDNA hybridization sites. The letter x indicates fluorescein precipitate.

weakly stained (Figs. 2*a* and 2*c*). The ratio of the largest to smallest chromosome within single metaphase cells was up to 4:1 and metacentric, sub-metacentric, subtelocentric, and telocentric chromosomes were visible, as reported previously (see Olin-Fatih and Heneen 1992).

Figure 2 shows metaphases before in situ hybridization; the same metaphase as Fig. 2*b* is shown in Fig. 6*a* after in situ hybridization. The loss of morphological definition in the chromosomes because of the denaturation and hybridization treatments is clear; in particular, primary constrictions at the centromeres become less visible. After in situ hybridization, the DAPI staining often showed a C-banding pattern, presumably because denaturation and washing selectively removes some DNA. Arrows on Fig. 2*b* indicate some negative bands that give DAPI-positive C-bands after hybridization (shown in Fig. 6*a*).

rDNA loci

After in situ hybridization with the rDNA probe, the number of loci of rDNA in each species could be determined by counting sites on metaphase chromosomes (Figs. 3–6) or interphase and prophase nuclei (Figs. 7–13). The number of major sites of hybridization observed in each species is presented in Fig. 1.

The three amphidiploid species and *B. campestris* had major and minor rDNA loci that varied in fluorescent intensity and size. When several metaphase and interphase nuclei of *B. nigra* and *B. oleracea* were examined, there was no regular pattern of large and small loci, although variation in sizes of loci was observed in some individual nuclei (Fig. 5). rDNA loci were present at characteristic terminal or interstitial locations along chromosome arms. Some were located next to major C-bands (Figs. 3–6).

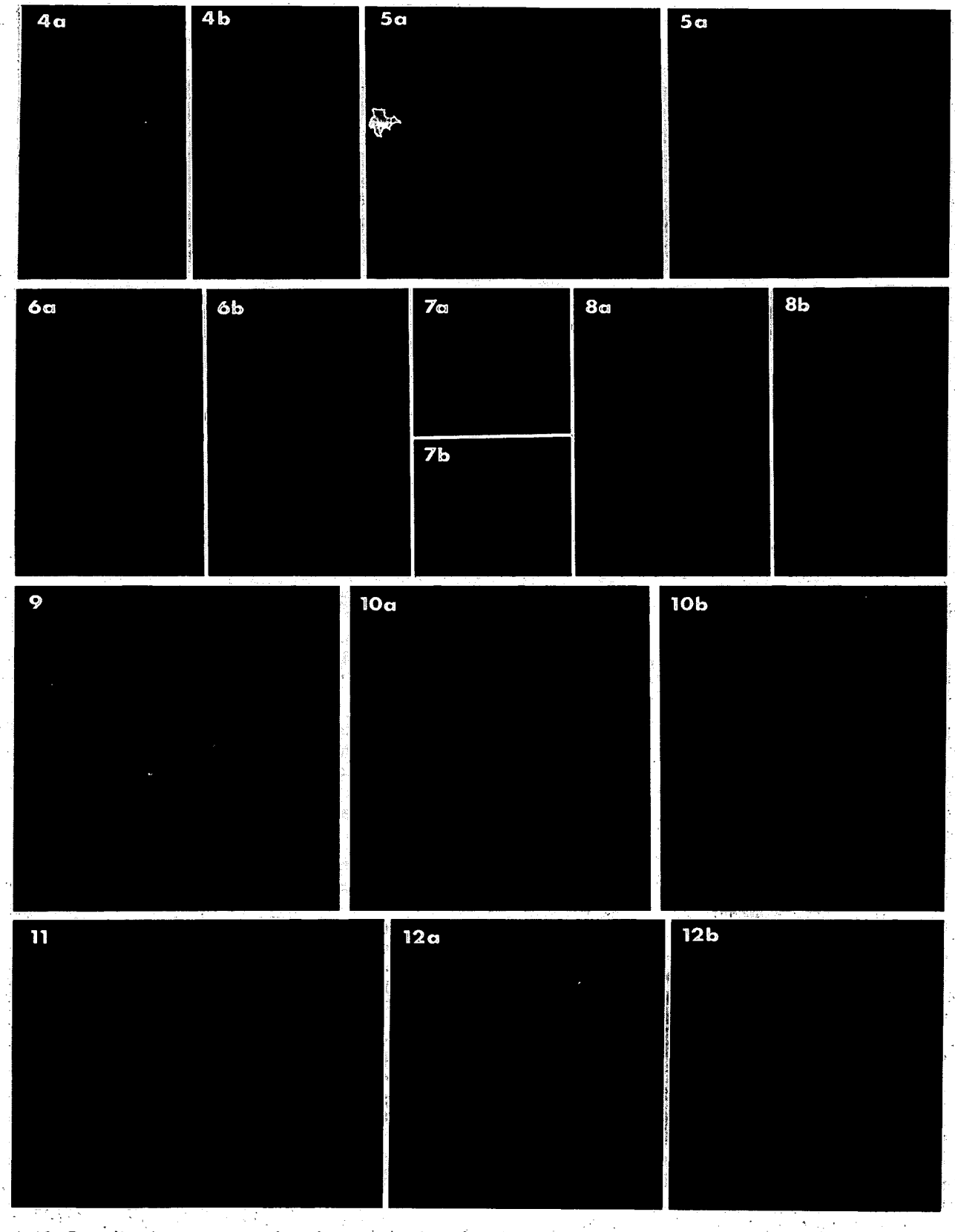
Interphase decondensation

Loci showed varying dispersion patterns during interphase. Sometimes, all rDNA loci were visible as discrete, large hybridization sites, with little hybridization within the nucleoli (Figs. 7–9). In other nuclei, there was dispersion or decondensation of the signal within nucleoli (Figs. 10–13).

Discussion

Cytology and in situ hybridization

DAPI staining and fluorescence microscopy showed the morphology of the metaphase chromosomes and the disposition of condensed and decondensed DNA during interphase (Figs. 2–13). DAPI stains AT-rich sequences more strongly than those that are GC rich (see Schweizer 1980), and some GC-rich regions occupy much of a chromosome arm. The centromeric regions of metaphase chromosomes tended to stain less strongly than the rest of the chromosomes, often corresponding to C-band positive regions (Figs. 2*b* and 6*a*; Olin-Fatih and Heneen 1992). The results show that a fluorochrome-banded karyotype



FIGS. 4–12. Root-tip chromosome and nuclear preparations from *Brassica* species. Blue staining is DAPI fluorescence showing DNA. Orange-red fluorescence shows DNA counterstaining with propidium iodide. Yellow-green fluorescence shows sites of in situ hybridization of digoxigenein-labelled rDNA, detected by fluorescein-labelled antibodies. The strength of orange-red staining (which also affected the blue colouring) was varied in different experiments to prevent hybridization sites being

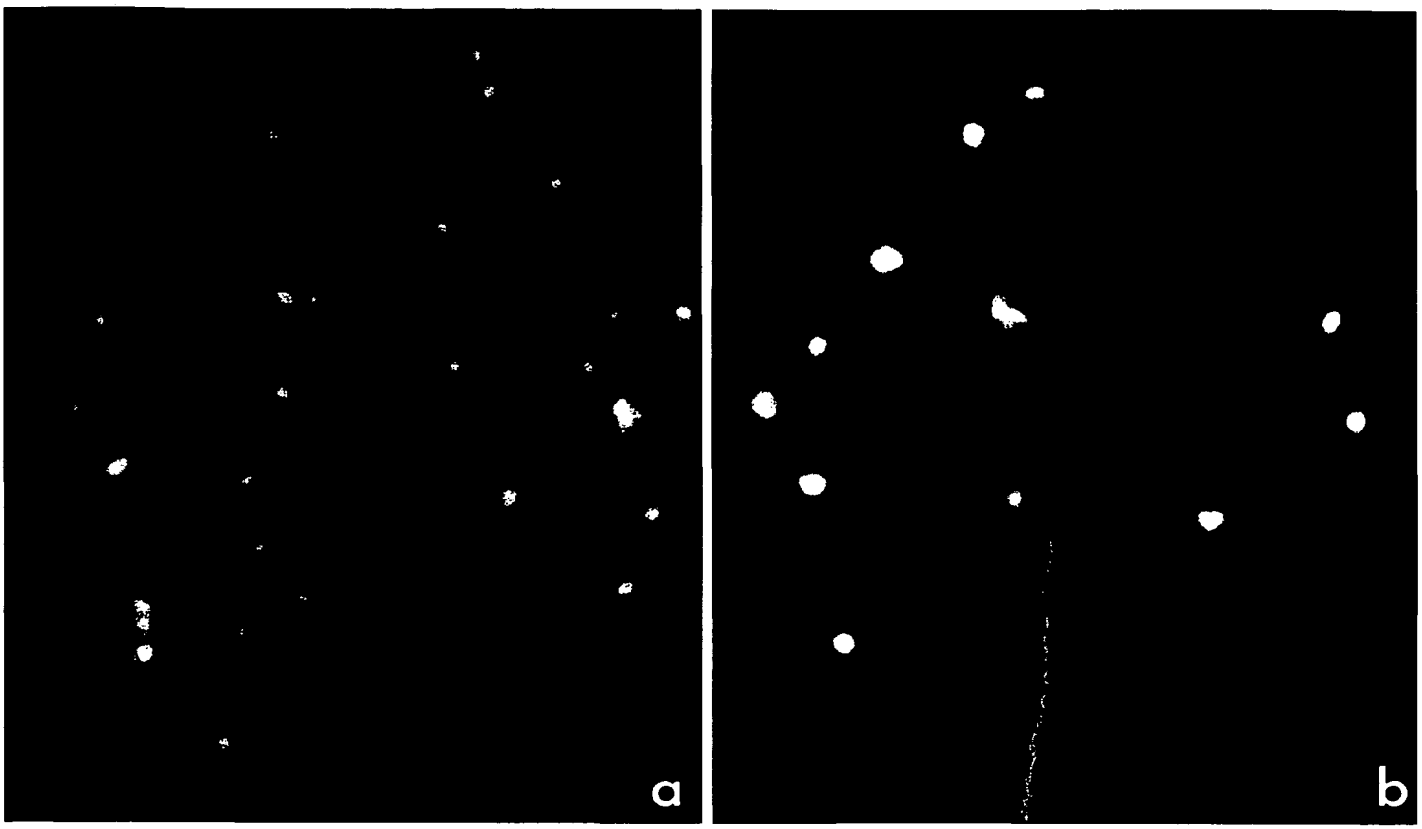


FIG. 13. *Brassica napus* prometaphase. (*a*) DAPI staining. (*b*) Six pairs of rDNA hybridization loci.

could be constructed; however, like the C-banded karyotype (Olin-Fatih and Heneen 1992), it is unlikely to identify unequivocally all the chromosome pairs in the tetraploid species and hence would not be of great value for analysis of aneuploid lines.

A GC-rich (58%) middle repetitive DNA sequence has been sequenced from *B. campestris* and shown to hybridize to centromeric regions in situ (Iwabuchi et al. 1991). Other clones that hybridize to centromeric regions of A- and C-genome chromosomes have also been identified (G.E. Harrison and J.S. Heslop-Harrison, in preparation; Heslop-Harrison et al. 1992). The similarity of fluorescence properties of centromeres of many chromosomes from the three genomes indicates that many have GC-rich sequences, although the Southern hybridization data indicates that these are not closely related to the sequence from *B. campestris* (Iwabuchi et al. 1991). Such sequences may be valuable for identifying the genome origin of chromosomes by in situ hybridization.

Propidium iodide staining is slightly stronger in the centromeric region (Fig. 6*b*) and next to the rDNA (see later). The sites of rDNA hybridization are generally less strongly DAPI stained than others (Figs. 4 and 6). These regions are often distended and sometimes connected (Fig. 6), presumably because of residual proteins and RNA associated with the activity of the locus at the preceding interphase. In DAPI-stained metaphases, such connected regions and chromatin strands are often seen (Fig. 2*a*, arrow). Major rDNA loci also appear slightly more red in DAPI fluorescence after in situ hybridization of the rDNA probe (e.d., Fig. 6*a*).

As in many other plant and animal species, fluorescence in situ hybridization was able to localize rDNA loci on chromosomes with good morphological preservation. In situ hybridization, like Southern hybridization, is not usually a fully quantitative technique, but large differences in hybridization signal strength at different loci correspond to major differences in copy number of the tandem repeat unit between rDNA loci. Absolute levels of sensitivity are difficult to quantify, but we can compare the results presented in the figures with results using similar techniques with single or low copy clones of known length and genomic distribution to *Arabidopsis thaliana* and *H. vulgare* (J. Maluszynska, G.E. Harrison, T. Schwarzacher, I.J. Leitch and J.S. Heslop-Harrison, unpublished). The comparison indicates that a minimum

obscured. Fig. 4. *Brassica campestris* metaphase. (*a*) DAPI. (*b*) Five pairs of rDNA loci. Fig. 5. *Brassica oleracea* metaphase. (*a*) DAPI. (*b*) Two pairs of rDNA loci. Fig. 6. *Brassica napus*. (*a*) DAPI. (*b*) Six pairs of rDNA loci. Stretched yellow hybridization in one region of the nucleus is presumably a remnant of the nucleolus that has remained in metaphase. Fig. 7. *Brassica nigra* interphase. (*a*) DAPI. (*b*) Two pairs of rDNA loci, all condensed. Fig. 8. *Brassica oleracea* interphase. Two pairs of rDNA loci, all condensed. Fig. 9. *Brassica campestris* interphase. Five pairs of rDNA loci, all condensed. Fig. 10. *Brassica carinata* interphase. (*a*) DAPI. (*b*) Eight major rDNA loci. Fig. 11. *Brassica juncea* interphase. Five major, and one minor, pairs of rDNA loci are visible, five of which are partially decondensed and run into the nucleolus. Fig. 12. *Brassica napus* interphase. Six pairs of rDNA loci.

of 50 copies of the rDNA repeat unit would be detectable as clear hybridization sites on both chromatids of both homologous chromosomes in every suitable metaphase, as obtained in the present series of experiments. Higher detection sensitivities might detect additional rDNA loci in *Brassica*; more minor loci, although they may be expressed, are probably of little genetic significance and repeat unit copy number can change relatively rapidly over generations (Flavell 1986).

rDNA loci: diploid species

Five rDNA loci were found in the diploid species *B. campestris* (Figs. 4*b* and 9). Röbbelen (1960) reported that *B. campestris* had two NOR-bearing chromosomes with characteristic morphology, which were both associated with nucleoli. Iwabuchi et al. (1991) used in situ hybridization to show strong hybridization to one chromosome pair and faint but reproducible signals on three other chromosome pairs using a clone of part of the 25S rRNA gene. Delseny et al. (1990) found no higher level of RFLP complexity (at least in the exposures of the Southern hybridization experiments shown) in the species than in other diploids, indicating that the five different loci are similar. However, they did find that the level of divergence of *B. campestris* (A genome) from *B. oleracea* (C genome) was higher than that between *B. nigra* (B genome) and *B. oleracea* based on rDNA subunit size and rDNA RFLP patterns.

The two other diploid species, *B. nigra* and *B. oleracea*, had two major pairs of rDNA loci when analyzed by in situ hybridization, and *B. oleracea* had an extra minor pair of loci (Fig. 5). These results agree with the conclusions of Röbbelen (1960) from analysis of associations of chromosomes with the nucleoli. Kianian and Quiros (1992*a*) identified three unlinked loci of rDNA in *B. oleracea* by *Eco*RI RFLP analysis using the same probe, pTa71. The number of rDNA carrying chromosomes can also be identified by their species-specific RFLPs in addition lines of *B. campestris–oleracea* (Delseny et al. 1990), *B. napus–nigra* (Chèvre et al. 1991), and *Diplotaxis erucoides – B. nigra* (This et al. 1990), and the results agree with those from in situ hybridization.

rDNA loci: amphidiploid species

Chromosome numbers in all three tetraploid species equal the sums of numbers in their ancestral diploid species (Fig. 1). The tetraploid species have fewer rDNA loci than the sum of their ancestors (Fig. 1). *Brassica juncea* (AABB genomes; Fig. 11) has five major pairs and one minor pair of rDNA loci, one less than the sum of those in its ancestors *B. nigra* (BB genome, 2 loci) and *B. campestris* (AA genome, 5 loci). RFLP analysis shows that *B. juncea* had rDNA RFLPs characteristic of both ancestors (Delseny et al. 1990). There were six major rDNA loci in *B. napus* (AACC genomes). Again, the species has all the restriction sites found in its diploid ancestors (Quiros et al. 1987). It is probable that the extra loci were lost (or are reduced to below the detection limit), although translocations and fusions could also lead to loss of sites. Bennett and Smith (1991) reported the total copy numbers of the *B. oleracea* and *B. campestris* types of rDNA, determined by quantitative Southern hybridization, as 800 and 3500 in the tetraploid *B. napus*, compared with 1500 and 3400 in the respective ancestral tetraploid species. The in situ hybridization data support these results, since the total strength of hybridization is lower in *B. oleracea* and *B. campestris* (cf. Figs. 5*b* and 8 with 4*b*, and 9). In *B. napus* (Figs. 6*b* and 13*b*), there is substantial varition in size of the individual rDNA loci; the Bennett and Smith (1991) results indicate that the smaller loci arise from *B. oleracea*.

Molecular cytogenetics of Brassica *species*

RFLP mapping data from diploid *Brassica* species suggested that extensive structural rearrangements of chromosomes have occurred after the duplication events (Slocum et al. 1990; Song et al. 1991; Kianian and Quiros 1992*b*). In both diploid and tetraploid species, differences in the organization of repetitive DNA sequences at the centromeres detected by in situ hybridization also indicate that structural rearrangements have occurred between species (G.E. Harrison and J.S. Heslop-Harrison, in preparation; see Heslop-Harrison et al. 1992). Although many rearrangements may have occurred during evolution of the tetraploid species from the diploids, genome size measurements by Feulgen microdensitometry (Bennett and Smith 1976; Fig. 1) and flow cytometry (Arumuganathan and Earle 1991), as well as the chromosome sizes found here (Figs. 2–6) and in other karyotype studies, also indicate that no major changes in average chromosome sizes have occurred in the amphidiploid species. Careful, systematic measurement of chromosome and genome sizes in the species may indicate that overall genome size is slightly different from the sum of the ancestors, but quantitative and qualitative assessment of differences in copy number, distribution, and actual sequence of various DNA sequence families are likely to be of greater importance to understanding microevolution (during selection in plant breeding, tissue culture, or the production of aneuploid lines) and macroevolution (during speciation) within the genus.

Molecular cytogenetic techniques offer exciting possibilities for chromosome investigations in the *Brassica*s with many small, similarly sized chromosomes, especially when combined with the extensive molecular genetic information now accumulating about the genus. For example, the use of double target in situ hybridization with genome-specific probes (Heslop-Harrison et al. 1992) and the rDNA probe is likely to be able to detect which genome has lost rDNA sites. In humans, sets of pooled chromosome-specific probes can be used for "painting" particular chromosomes along their lengths by in situ hybridization (Lichter et al. 1988). Perhaps similar probe sets could be derived from *Arabidopsis thaliana* ($2n = 10$) where no duplicated segments have been reported and used to paint the homologous segments of the diploid and tetraploid *Brassica* species. Evolutionary translocations and other rearrangements could then be visualized and analyzed directly. Knowledge of the types of changes that have happened in the past may be a valuable guide to the changes (Heslop-Harrison and Schwarzacher 1993) that can be made during plant breeding using genes present wihin the cultivated *Brassica*'s and their wild relatives.

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Arumuganathan, K., and Earle, E.D. 1991. Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. **9**: 229–233.

Bennett, M.D., and Smith, J.B. 1976. Nuclear DNA amounts in angiosperms. Phil. Trans. R. Soc. Lond. B, **274**: 227–274.

Bennett, R.I., and Smith, A.G. 1991. The complete nucleotide sequence of the intergenic spacer region of an rDNA operon from *Brassica oleracea* and its comparison with other crucifers. Plant Mol. Biol. **16**: 1095–1098.

Chèvre, A.M., This, P., Eber, F., et al. 1991. Characterization of disomic addition lines *Brassica napus – Brassica nigra* by isozyme, fatty acid, and RFLP markers. Theor. Appl. Genet. **81**: 43–49.

Delseny, M., McGarth, J.M., This, P., Chèvre, A.M., and Quiros, C.F. 1990. Ribosomal RNA genes in diploid and amphiploid *Brassica* and related species: organization, polymorphism, and evolution. Genome, **33**: 733–744.

Flavell, R.B. 1986. The structure and control of expression of ribosomal RNA genes. Oxf. Surv. Plant Mol. Cell Biol. **3**: 252–274.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Heslop-Harrison, J.S. 1991. The molecular cytogenetics of plants. J. Cell Sci. **100**: 15–21.

Heslop-Harrison, J.S., and Schwarzacher, T. 1993. Molecular cytogenetics—biology and applications in plant breeding. Chromosomes Today, **11**: 191–198.

Heslop-Harrison, J.S., Schwarzacher, T., Anamthawat-Jónsson, K., Leitch, A.R., Shi, M., and Leitch, I.J. 1991. In situ hybridization with automated chromosome denaturation. Technique, **3**: 109–115.

Heslop-Harrison, J.S., Harrison, G.E., and Leitch, I.J. 1992. Reprobing of DNA:DNA in situ hybridization preparations. Trends Genet. **8**: 372–373.

Iwabuchi, M., Itoh, K., and Shimamoto, K. 1991. Molecular and cytological characterization of repetitive DNA sequences in *Brassica*. Theor. Appl. Genet. **81**: 349–355.

Kianian, S.F., and Quiros, C.F. 1992*a*. Genetic analysis of major multigene families in *Brassica oleracea* and related species. Genome, **35**: 516–527.

Kianian, S.F., and Quiros, C.F. 1992*b*. Generation of a *Brassica oleracea* composite RFLP map: linkage arrangements among various populations and evolutionary implications. Theor. Appl. Genet. **84**: 544–554.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by *in situ* hybridization. Genome, **35**: 1013–1018.

Leitch, A.R., Schwarzacher, T., Wang, M.L., et al. 1993. Molecular cytogenetic analysis of repeated sequences in a long term wheat suspension culture. Plant Cell, Tissue Organ Cult. **33**: 287–296.

Lichter, P., Cremer, T., Borden, J., Manuelidis, L., and Ward, D.C. 1988. Delineation of individual human chromosomes in metaphase and interphase cells by *in situ* suppression hybridization using recombinant DNA libraries. Hum. Genet. **80**: 224–234.

Maluszynska, J., and Heslop-Harrison, J.S. 1991. Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J. **1**: 159–166.

Maluszynska, J., and Heslop-Harrison, J.S. 1993. Molecular cytogenetics of the genus *Arabidopsis*: *in situ* localization of rDNA sites, chromosome numbers and diversity in centromeric heterochromatin. Ann. Bot. **71**: 479–484.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 479–497.

Olin-Fatih, M., and Heneen, W.K. 1992. C-banded karyotypes of *Brassica campestris*, *B. oleracea*, and *B. napus*. Genome, **35**: 583–589.

Prakash, S., and Chopra, V.L. 1991. *In* Chromosome engineering in plants: genetics, breeding, evolution. Part B. *Edited by* T. Tsuchiya and P.K. Gupta. Elsevier, Amsterdam. pp. 161–180.

Quiros, C.F., Ochoa, O., Kianian, S.F., and Douches, D. 1987. Analysis of the *Brassica oleracea* genome by the generation of *B. campestris–oleracea* chromosome addition lines: characterization by isozymes and rDNA genes. Theor. Appl. Genet. **74**: 758–766.

Röbbelen, G. 1960. Beiträge zur Analyse des *Brassica*-Genomes. Chromosoma, **11**: 205–228.

Schwarzacher, T., Ambros, P., and Schweizer, D. 1980. Application of Giemsa banding to orchid karyotype analysis. Plant Syst. Evol. **134**: 293–297.

Schweizer, D. 1980. Fluorescent chromosome banding in plants: applications, mechanisms, and implications for chromosome structure. The plant genome. *Edited by* D.R. Davies and D.A. Hopwood. John Innes Charity, Norwich. pp. 61–72.

Slocum, M.K., Figdore, S.S., Kennard, W.C., Suzuki, J.Y., and Osborn, T.C. 1990. Linkage arrangement of restriction fragment length polymorphism loci in *Brassica oleracea*. Genome, **80**: 57–64.

Song, K.M., Suzuki, J.Y., Slocum, M.K., Williams, P.H., and Osborn, T.C. 1991. A linkage map of *Brassica rapa* (syn. *campestris*) based on restriction fragment length polymorphism loci. Theor. Appl. Genet. **82**: 296–304.

This, P., Ochoa, O., and Quiros, C.F. 1990. Dissection of the *Brassica nigra* genome by monosomic addition lines. Plant Breed. **105**: 211–220.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn. J. Bot. **7**: 389–452.

Direct and sensitive fluorescence in situ hybridization of 45S rDNA on tomato chromosomes

JIE XU[1] AND E.D. EARLE

Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853-1902, U.S.A.

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We describe a direct and sensitive fluorescence in situ hybridization protocol for plant chromosomes. We labelled 45S rDNA with fluorescein-12-dUTP and hybridized to somatic chromosomes of four tomato genotypes. This technique does not require posthybridization immunocytochemical amplifications. The improved signal sensitivity with this technique allowed identification of new rDNA loci on three pairs of chromosomes, in addition to the previously known locus on chromosome 2. We discuss favorable features of direct fluorescence in situ hybridization for chromosomes fixed on a slide and chromosomes or cells in suspension.

Key words: direct fluorescence in situ hybridization, 45S rDNA, tomato chromosomes.

XU, J., et EARLE, E.D. 1994. Direct and sensitive fluorescence in situ hybridization of 45S rDNA on tomato chromosomes. Genome, **37** : 1062–1065.

Les auteurs décrivent un protocole direct et sensible de détection par fluorescence d'hybridation in situ pour les chromosomes de plantes. L'ADN ribosomique de 45S a été marqué avec de la fluorescéine-12-dUTP et hybridé avec des chromosomes somatiques de quatre génotypes de tomate. Cette technique ne requiert aucune amplification immunocytochimique en post-hybridation. L'augmentation de la sensibilité dans les signaux en employant cette technique a permis d'identifier de nouveaux loci d'ADN ribosomique répartis sur trois paires de chromosomes en sus du locus déjà identifié sur le chromosome 2. Les auteurs discutent des caractéristiques favorables de la fluorescence d'hybridation in situ directe pour les chromosomes fixés sur lames et pour l'observation de chromosomes ou de cellules en suspension.

Mots clés : fluorescence d'hybridation in situ, ADNr 45S, chromosomes de la tomate.

[Traduit par la rédaction]

Introduction

Applications of fluorescence in situ hybridization (FISH) techniques to plant chromosomes play an increasingly important role in plant molecular cytogenetics and chromosome engineering. Depending on DNA probes used, a genome (Schwarzacher et al. 1989; Xu et al. 1994), individual chromosome or chromosome segment (Schwarzacher et al. 1992), repetitive or low-copy DNA sequences (Mukai et al. 1993), or genes (Lehfer et al. 1993) can be highlighted selectively. Multicolor FISH permits simultaneous detection of two or more probes in a single experiment (Leitch et al. 1991; Mukai et al. 1993).

The conventional FISH protocols employ posthybridization immunocytochemical detection and sometimes several rounds of amplifications to visualize hybridization signals. Although these indirect FISH methods can effectively generate hybridization signals, the posthybridization amplifications are laborious and time-consuming and have the potential for high background noise. In human cytogenetics, several reports on the utility of DNA probes labelled with fluorochromized dUTPs in direct FISH of human chromosomes illustrate improved resolution of signals (Wiegant et al. 1991, 1993). The presence of cell wall and cytoplasmic debris in plant chromosome preparations not only makes it difficult for a DNA probe to penetrate into target chromosomal sequences but also increases the chance of nonspecific binding of antibodies used in immunocytochemical detection procedures, thus reducing signal detection sensitivity. This, together with low mitotic index inherent in plant cells, makes FISH less efficient for plant chromosomes than for human or animal chromosomes. Simplified and sensitive FISH protocols are needed for plant chromosome studies.

In this paper, we report direct FISH of tomato chromosomes using fluorescein-12-dUTP labelled 45S rDNA as a probe. The high sensitivity of the technique enabled new rDNA loci to be clearly identified.

Materials and methods

Plant materials and chromosome preparations

Three cultivars of tomato (*Lycopersicon esculentum* Mill.), 'Rio Grande', 'VF 36', and 'Motelle' ($2n = 2x = 24$) and a primary trisomic 2 ($2n = 2x + 1 = 25$) were used. 'Rio Grande', 'VF 36', and primary trisomic 2 were kindly provided by C.M. Rick, University of California, Davis, and 'Motelle', by A. Frary, Cornell University. Root tips from plants were treated with 0.1% colchicine for 3–4 h at 12°C and fixed in ethanol – glacial acetic acid (3:1). The root tips were squashed in 5% acetocarmine (Carolina Biological Supply Company) and coverslips were removed after freezing in liquid nitrogen. The slides were destained in 45% acetic acid for 5–7 min and dehydrated in 95% ethanol for 10 min at room temperature. They were then oven-dried for 2–5 h at 37°C before in situ hybridization.

DNA probe

The plasmid used as the probe was provided by K. Arumuganthan, University of Nebraska. It contains a 9.1-kb 45S rDNA insert that was isolated from tomato and cloned in the vector pUC 18 by P. Palukaitis, Cornell University. The insert includes the 5.8S,

[1]Author to whom all correspondence should be addressed.

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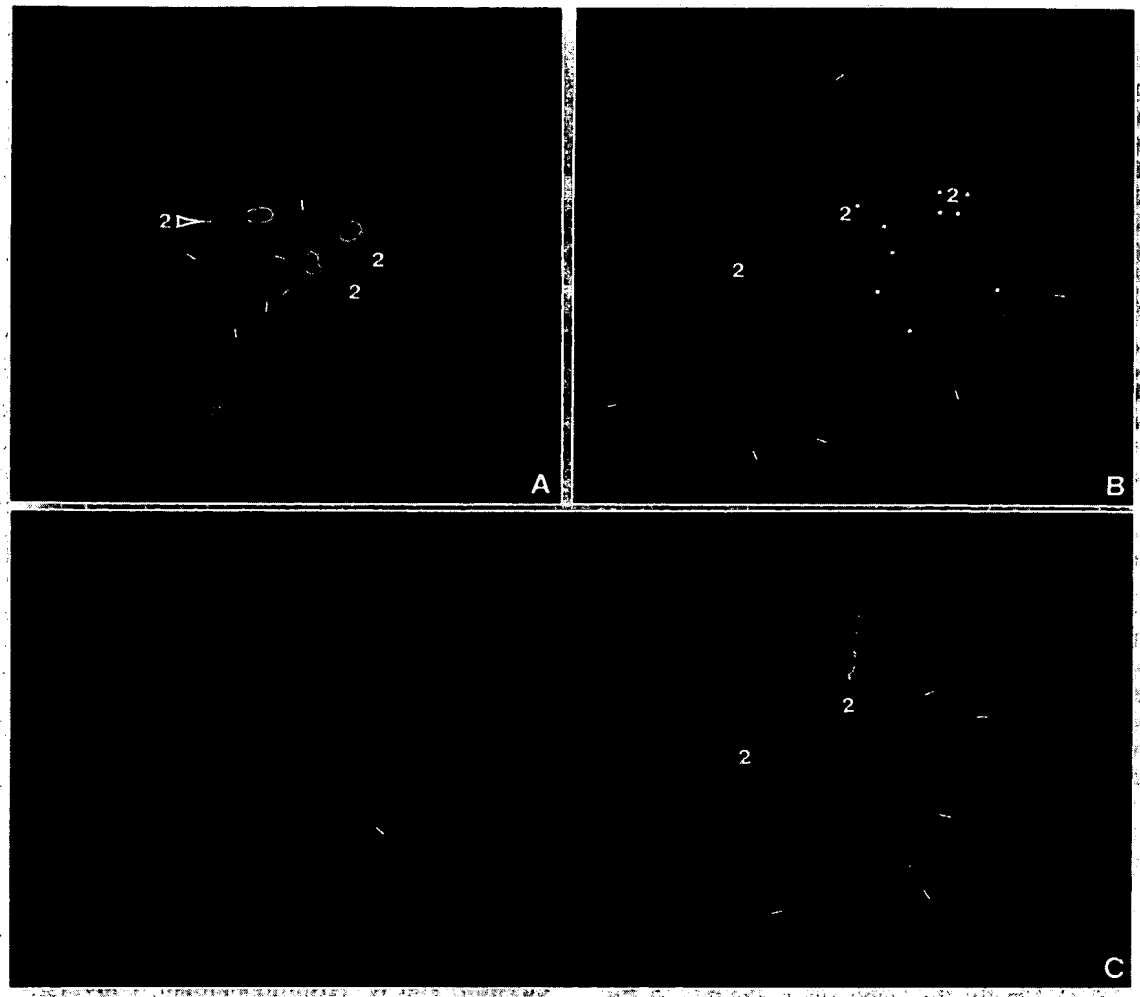

FIG. 1. Fluorescence in situ hybridization pattern of 45S rDNA in tomato somatic chromosomes (×1000). Yellow–green hybridization sites are seen in chromosome 2 and six (three pairs) other chromosomes (pointed with white bars). (A and B) Trisomic 2 ($2n = 2x + 1 = 25$) with three hybridized satellites, of which (A) all attached to chromosome 2's in a highly condensed chromosome and (B) two separated from chromosome 2's with each satellite and its bearing chromosome being identified by light yellow–green connecting track and indicated with six dots or four stars. (C) Tomato ('Rio Grande', $2n = 2x = 24$) somatic cell at late prophase with less condensed chromosomes and visible nucleolus, showing a pair of satellites painted plus three additional pairs of chromosomes with hybridization signals.

18S, and 25S rDNA subunits and nontranscribed spacer sequences. The plasmid was labelled with fluorescein-12-dUTP by nick translation with a 1:1.6 ratio of fluorescein-12-dUTP to dTTP (Boehringer Mannheim). After nick translation, the probe was precipitated with ethanol.

In situ hybridization

Chromosomal DNA on the slides was denatured in 70% formamide in 2× SSC (0.15 M NaCl, 0.015 M sodium citrate) for 2 min at 70°C and dehydrated through an ethanol series at −20°C. For each slide, 10 μL of hybridization mixture (50% formamide, 10% dextran sulfate, 2× SSC, 50 ng of mechanically sheared salmon sperm DNA, and 20–40 ng of fluorescein-12-dUTP labelled probe) was used. The probe mixture was denatured for 10 min at 80°C and loaded onto the slides. A coverslip was added and sealed with rubber cement. The slides were redenatured for 5–7 min at 80°C. After overnight hybridization in a humid chamber at 37°C, the slides were washed two times for 5–7 min each in 50% formamide in 2× SSC and then two times for 5 min each in 2× SSC, all at 40°C. The slides were then washed two times for 10 min each in PN buffer (0.1 M NaH_2PO_4–Na_2HPO_4, 0.1% Nonidet P-40, pH 8.0) at room temperature. Antifade Vectashield mounting medium (Vector Laboratories) containing 2–5 μg/mL of propidium iodide (PI) was used to mount the slides and counterstain chromosomes. Slides were examined with an Olympus Vanox AHBT fluorescence microscope with a blue excitation filter. Photographs were taken on Kodak print film ISO 1600. More than 10 well-spread metaphase cells were studied for each of the four genotypes.

In addition, pUC 18 alone was labelled with fluorescein-12-dUTP and used in the FISH. No hybridization signals were detected in any of the metaphase and interphase cells examined, confirming absence of homology between the vector sequence and tomato chromosomal DNA.

Results

The protocol does not need immunocytochemical amplifications to visualize hybridization signals. Figure 1 shows fluorescence microphotographs of tomato somatic chromosomes at metaphase (Figs. 1A and 1B) or at late prophase (C). Hybridization signals are seen as yellow–green against orange–red chromosomes counterstained with PI. Tomato chromosome 2 is characterized by having a secondary

constriction, corresponding to the nucleolus organizer region (NOR), at the short arm (Vallejos et al. 1986; Ganal et al. 1988). Figure 1A shows a trisomic 2 metaphase cell with highly condensed chromosomes and three chromosomes 2 painted yellow–green over about half the length of the chromosomes, including the end of the short arm, NOR, and entire satellite. This observation confirms the origin of the rDNA sequence (Vallejos et al. 1986). In addition, six other chromosomes (presumably three pairs) have a hybridization site (indicated with bars, also in Figs. 1B and 1C). Figure 1B shows another trisomic metaphase cell where two of three chromosomes 2 have satellites separated from the short arm, probably as a result of expression of the genes (Gustafson et al. 1988; Mukai et al. 1991). The light yellow–green fluorescent track connecting the satellite and short arm enabled the satellited chromosomes to be identified.

Three tomato cultivars ('Rio Grande', 'VF 36', and 'Motelle') all have a pair of chromosomes 2 with satellites entirely painted and three other pairs of chromosomes with a hybridization signal (Fig. 1C).

Discussion

The present experiment demonstrates the application of direct FISH on a plant species (tomato) having small chromosomes. The technique has the following favorable features. First, the omission of several labor-intensive posthybridization washing and amplification steps not only saves 3–4 h, but it also reduces the likelihood of loss of, or damage to, chromosomes during the process. Second, the technique produces less nonspecific noise and better contrast between hybridization signals and chromosome and cytoplasmic background. Good resolution obtained with the technique allows clear detection of additional hybridization sites even in highly condensed chromosomes (Fig. 1A). In human chromosomes, the detection sensitivity of direct FISH was found to be 50–100 kb (Wiegant et al. 1991). Finally, the technique is flexible. A fluorochromized dUTP can readily be incorporated into a probe using nick translation, random priming, or PCR. Different colored fluorescent dUTPs that are commercially available can be applied for direct and multicolor FISH (Wiegant et al. 1993). When necessary, the hybridization signals can be amplified using immunocytochemical detection reagents.

The 45S rDNA sequence was estimated in approximately 2300 copies in the tomato genome (Vallejos et al. 1986). Based on RFLPs between *L. pennelli* and *L. esculentum*, Vallejos et al. (1986) placed the 45S rDNA locus at a distal point on the short arm of chromosome 2. By in situ hybridization using a biotinylated probe and enzyme-mediated detection system, Ganal et al. (1988) observed that the sequence hybridized strongly to the end of an acrocentric chromosome, presumably chromosome 2, of tomato 'VF 36'. In the present experiment we used trisomic 2 to verify the identity of the rDNA sequence. Our finding of new rDNA loci in three pairs of chromosomes in all four genotypes examined, including 'VF 36, is probably a result of an improved detection sensitivity with the direct FISH over the traditional in situ protocol used by Ganal et al. (1988). Failure to detect the additional rDNA loci using RFLPs is probably due to the limited markers used by Vallejos et al. (1986). They used 18 enzyme markers defining 10 of 12 tomato chromosomes, averaging less than two markers per chromosome. Further work using the direct FISH of the probe on a set of tomato trisomics should identify which chromosomes carry these loci.

We noticed that the rDNA probe heavily hybridized to the entire satellite (Fig. 1) in addition to the secondary constriction (Fig. 1A) in chromosome 2. This hybridization pattern is similar to that in *Allium* (Ricroch et al. 1992) and *Aegilops squarrosa* (Mukai et al. 1991) but different from that in rye (Gustafson et al. 1988), wheat (Mukai et al. 1991), and barley (Xu and Kasha 1992) where the probe was mainly hybridized to the secondary constrictions in satellited chromosomes. Presence of new and minor rDNA loci in chromosomes in addition to satellited chromosomes was revealed by in situ hybridization techniques in many plant species including wheat (Mukai et al. 1991), barley (Leitch and Heslop-Harrison 1992), *Allium* (Ricroch et al. 1992), and white spruce (Brown et al. 1993).

The technique we describe in this paper could be fine tuned for direct FISH of cells or chromosomes in suspension. Indirect FISH of cell suspensions has been used for studies of gene expression (Timm and Stewart 1992) and for purification by sorting of a cell population expressing a specific mRNA (Lalli et al. 1992). FISH of chromosome suspensions using a collection of chromosome-specific probes should preferentially highlight the target chromosome. This should provide a basis for flow cytometry to sort out the target chromosome for construction of a chromosome-enriched library. By eliminating immunocytochemical amplification steps, the direct FISH will not only simplify the process but also minimize damage to cells or chromosomes caused by repeated centrifugations and resuspensions needed in indirect FISH procedures.

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Brown, G.R., Amarasinghe, V., Kiss, G., and Carlson, J.E. 1993. Preliminary karyotype and chromosomal localization of ribosomal DNA sites in white spruce using fluorescence in situ hybridization. Genome, **36**: 310–316.

Ganal, M.W., Lapitan, N.L.V., and Tanksley, S.D. 1988. A molecular and cytogenetic survey of major repeated DNA sequences in tomato (*Lycopersicon esculentum*). Mol. Gen. Genet. **213**: 262–268.

Gustafson, J.P., Dera, A.R., and Petrovic, S. 1988. Expression of modified rye ribosomal RNA genes in wheat. Proc. Natl. Acad. Sci. U.S.A. **85**: 3943–3945.

Lalli, E., Gibellini, D., Spartaco, S., and Facchini, A. 1992. In situ hybridization in suspension and flow cytometry as a tool for the study of gene expression. Anal. Biochem. **207**: 298–303.

Lehfer, H., Busch, W., Martin, R., and Herrmann, R.G. 1993. Localization of the B-hordein locus on barley chromosomes using fluorescence in situ hybridization. Chromosoma, **102**: 428–432.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by in situ hybridization. Genome, **35**: 1013–1018.

Leitch, I.J., Leitch, A.R., and Heslop-Harrison, J.S. 1991. Physical mapping of plant DNA sequences by simultaneous in situ hybridization of two differently labelled fluorescent probes. Genome, **34**: 329–333.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping

of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.
Mukai, Y., Nakahara, Y., and Yamamoto, M. 1993. Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome, **36**: 489–494.
Ricroch, A., Peffley, E.B., and Baker, R.J. 1992. Chromosomal location of rDNA in *Allium* in situ hybridization using biotin and fluorescein-labelled probe. Theor. Appl. Genet. **83**: 413–418.
Schwarzacher, T., Leitch, A.R., Bennett, M.D., and Heslop-Harrison, J.S. 1989. In situ localization of parental genomes in a wide hybrid. Ann. Bot. (London), **64**: 315–324.
Schwarzacher, T., Anamthawat-Jonsson, K., Harrison, G.E., Islam, A.K.M.R., Jia, J.Z., King, I.P., Leitch, A.R., Miller, T.E., Reader, S.M., Rogers, W.J., Shi, M., and Heslop-Harrison, J.S. 1992. Genomic in situ hybridization to identify alien chromosomes and chromosome segments in wheat. Theor. Appl. Genet. **84**: 778–786.
Timm, E.A., Jr., and Stewart, C.C. 1992. Fluorescent in situ hybridization en suspension (FISHES) using digoxigenin-labeled probes and flow cytometry. BioTechniques, **12**: 362–367.
Vallejos, C.E., Tanksley, S.D., and Bernatzky, R. 1986. Localization in tomato genome of DNA restriction fragments containing sequences homologous to the rRNA (45S), the major chlorophyll a/b binding polypeptide and the ribulose bisphosphate carboxylase genes. Genetics, **112**: 93–105.
Wiegant, J., Reid, T., Nederlof, P.M., van der Ploeg, M., Tanke, H.J., and Raap, A.K. 1991. In situ hybridization with fluoresceinated DNA. Nucleic Acids Res. **19**: 3237–3240.
Wiegant, J., Wiesmeijer, C.C., Hoovers, J.M.N., Schuuring, E., d'Azzo, A., Vrolijk, J., Tanke, H.J., and Raap, A.K. 1993. Multiple and sensitive fluorescence in situ hybridization with rhodamine-, fluorescein-, and coumarine-labelled DNAs. Cytogenet. Cell Genet. **63**: 73–76.
Xu, J., and Kasha, K.J. 1992. Identification of a barley chromosomal interchange using N-banding and in situ hybridization techniques. Genome, **35**: 392–397.
Xu, J., Conner, R.L., and Laroche, A. 1994. C-banding and fluorescence in situ hybridization studies of wheat–alien hybrid 'Agrotana'. Genome, **37**: 477–481.

The distribution of cDNA sequences on field bean chromosomes

A. HOUBEN, A. BRANDES, AND I. SCHUBERT[1]
Institut für Pflanzengenetik und Kulturpflanzenforschung, D-06466 Gatersleben, Germany

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Total polyadenylated RNA was isolated from different organs of the field bean by oligo-dT latex particles. Complementary DNA (cDNA) was reverse transcribed from the mRNAs and used as a probe for fluorescent in situ hybridization to metaphase chromosomes. Hybridization signals covered the whole chromosome complement except for late-replicating Giemsa-banded heterochromatin regions, which are composed mainly (or exclusively) of tandemly arranged repetitive sequences.

Key words: cDNA, field bean, fluorescent in situ hybridization.

HOUBEN, A., BRANDES, A., et SCHUBERT, I. 1994. The distribution of cDNA sequences on field bean chromosomes. Genome, **37** : 1065–1067.

L'ARN polyadénylé total de différents organes du haricot de grande culture a été isolé à l'aide de particules de latex oligo-dT. L'ADN complémentaire (ADNc) s'est avéré transcrit de façon inversée à partir de l'ARNm et il a servi de sonde pour l'hybridation fluorescente in situ sur les chromosomes en stade métaphasique. Les signaux d'hybridation ont couvert le complément chromosomique entier, sauf pour les répliques tardives des bandes Giemsa des régions hétérochromatiques, lesquelles sont composées principalement (ou exclusivement) de séquences répétitives organisées en tandem.

Mots clés : ADNc, haricot de grande culture, hybridation fluorescente in situ.

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Introduction

Heterochromatin and euchromatin are present in all tested eukaryotic nuclear genomes and became visualized by different cytogenetic methods such as cold treatment, various Giemsa- and fluorescence-banding techniques, and by differential incorporation of base analogues. Heterochromatin usually proved to be genetically inert and mainly composed of satellite or highly repetitive simple sequence DNA. Euchromatin contains actively transcribed unique or low copy sequences in addition to dispersed repetitive ones. For detection of middle or high repetitive sequences, in situ hybridization is a reliable technique, but the number of single or low copy genes that were detectable on plant chromosomes by this method is rather small (Ambros et al. 1986; Gustafson et al. 1990; Lehfer et al. 1993; Leitch and Heslop-Harrison 1993).

[1]Corresponding author

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Fluorescent *in situ* hybridization to soybean metaphase chromosomes

Matthew C. Griffor, Lila O. Vodkin*, Ram J. Singh and Theodore Hymowitz
*Department of Agronomy, University of Illinois, Urbana, IL 61801, USA (*author for correspondence)*

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Abstract

Repetitive DNA sequences were detected directly on somatic metaphase chromosome spreads from soybean root tips using fluorescent *in situ* hybridization. Methods to spread the forty small metaphase chromosomes substantially free of cellular material were developed using protoplasts. The specific DNA probe was a 1.05 kb internal fragment of a soybean gene encoding the 18S ribosomal RNA subunit. Two methods of incorporating biotin residues into the probe were compared and detection was accomplished with fluorescein-labeled avidin. The rDNA probe exhibits distinct yellow fluorescent signals on only two of the forty metaphase chromosomes that have been counterstained with propidium iodide. This result agrees with our previous analyses of soybean pachytene chromosomes [27] showing that only chromosome 13 is closely associated with the nucleolus organizer region. Fluorescent *in situ* hybridization with the rDNA probe was detected on three of the forty-one metaphase chromosomes in plants that are trisomic for chromosome 13.

Introduction

In situ hybridization techniques developed in recent years are an important tool for the detection of specific nucleic acid sequences directly within cells or on chromosomes. Initially, *in situ* hybridization was used for the localization of DNA sequences on *Drosophila* polytene chromosomes or highly repeated sequences on metaphase chromosomes of diploid cells. In the past ten years, numerous refinements of the detection procedure have made possible the rapid, sensitive detection of short unique DNA sequences directly on mammalian metaphase chromosomes [2, 4, 13, 15, 16, 31]. *In situ* hybridization is a viable method for investigation of the spatial order of genes in interphase nuclei [15], the mechanism of gene amplification [30], and the identification of foreign DNA (transformed or viral) within animal and plant cells [8, 14].

The use of *in situ* hybridization in plants lags considerably behind its applications in cytogenetics of human and other animal systems. A major factor contributing to the difficulty in plants is obtaining mitotic and meiotic chromosomes free of cell wall material. Hybridization to plant metaphase chromosomes is often impeded due to their highly condensed nature. Difficulties are also encountered in the chromosome karyotyping of many plants including soybean, *Glycine max*. Soybean contains $2n = 40$ small (1.2–2.84 μm) morphologically similar mitotic metaphase chromosomes [25]. Despite its considerable economic importance, soybean lacks a cytogenetic map and a karyotype of all 20 soybean chromosomes has only recently been constructed for the relatively

uncondensed pachytene chromosomes [27]. In this report, we demonstrate the first application of *in situ* hybridization directly to individual soybean metaphase chromosomes.

Materials and methods

Plant material

Root tips from *Glycine max* (L) Merr. cv. Williams were used as the source for metaphase chromosomes. Seed of the primary trisomic S (satellited chromosome 13) line in soybean cv. Hodgson [24] were obtained from Reid Palmer, USDA/ARS, Agronomy Department, Iowa State University.

Chromosome preparation

Root tips were collected from secondary roots of seven-day-old seedlings grown in a sandbox in a greenhouse and pretreated for 2 to 4 h in 0.05% 8-hydroxyquinoline at 15 °C in order to obtain cells with a higher mitotic index. Root tips were then fixed in freshly prepared methanol, acetic acid, and chloroform (3 : 1 : 1), stored at − 20 °C, and used within 1 to 2 weeks for optimal results. Root tips were washed thoroughly in 0.01 M sodium citrate-citric acid (SC-CA) buffer at pH 4.6. Cell walls were digested in 2% cellulase ('Onozuka' R10) and 1% pectinase (Sigma) in SC-CA buffer for 2 h at 37 °C. Root tips were aspirated several times through a siliconized pasteur pipette to facilitate digestion of cell wall material. After enzymatic treatment, cells were subjected to a hypotonic treatment in 75 mM KCl for 10 min. A final fixation in the above fixative was performed for one hour on ice. Cells were dropped from a pasteur pipette onto methanol-cleaned and cold (− 70 °C) slides to promote chromosome spreading and were dried overnight at room temperature. Prepared slides were stored at − 70 °C in a vacuum-sealed bag and used within one week.

Probe and labeling methods

The soybean 18S rDNA probe pSR1.2B3 [3] contains a 1.05 kb internal fragment of the full 1807 bp rDNA gene and was supplied by R. Meagher, University of Georgia, Athens. Probe DNA was directly labeled by nick translation with bio-11-dUTP (Enzo Biochemical, New York) as per instructions from the supplier, resulting in incorporation levels of 30 to 50% bio-11-dUTP. Probe fragment size was controlled by adjusting the concentration of DNAse in the nick translation reaction to yield fragments between 300 to 1000 nucleotides. Fragment size was determined using alkaline gel electrophoresis followed by transfer to nitrocellulose and was visualized with strepavidin-conjugated alkaline phosphatase (Bethesda Research Laboratories). An indirect labeling method consisting of poly(T)-tailing DNAse-digested probe DNA was also employed. Detection of poly(T)-tailed probe was accomplished with a biotin-labeled poly(dA) molecule (BioBridge, from Enzo Biochemical, New York).

In situ hybridization

In situ hybridization was performed as described by Lawrence *et al.* [15] with modifications. Slides were treated with 100 μg/ml RNAse A (Sigma) in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 30 min at 37 °C and then sequentially dehydrated in an ethanol series of 70%, 95%, and 100% ethanol at room temperature. Slides were incubated in 0.1 M triethanolamine, pH 8.0, and 0.25% acetic anhydride for 10 min followed by denaturation in 70% formamide, 2 × SSC, pH 7.0, at 70 °C for 150 s and then immediately dehydrated 5 min each in 70%, 95%, and 100% ethanol at − 20 °C. They were then treated with 100 to 300 ng/ml proteinase K in 20 mM Tris-HC, 2 mM $CaCl_2$, pH 7.5, at 37 °C for 5 to 7.5 min and dehydrated as above. Probe and calf thymus DNA were denatured at 95 °C for 10 min, quick-cooled on ice, and then added to a hybridization mix. The final concentrations in the mix were 50% formamide,

2 × SSC, 10% dextran sulfate, 500 μg/ml calf thymus DNA, and 1 μg/ml denatured probe DNA. Of this mix 20 μl was applied to each slide under a 22 mm × 30 mm glass coverslip and sealed with rubber cement. Slides were incubated for 3 to 6 h in a humid chamber at 37 °C. After hybridization, slides were washed 2 times, 10 min each, in 50% formamide, 2 × SSC, pH 7, at 42 °C; 2 times, 5 min each, in 2 × SSC, pH 7, at 42 °C; and once in 1 × SSC, pH 7, for 10 min at room temperature. Slides were placed in PN buffer (0.1 M sodium phosphate, 0.05% Nonidet P-40, pH 8). Slides were not allowed to dry after this point. When poly(T)-tailed probe was used, 35 μl of a 1 : 50 dilution of the *BioBridge* labeling molecule in PN buffer was applied to each slide, covered with a parafilm coverslip, and incubated 10 min at room temperature. Slides were washed 3 times, 10 min each, in PN buffer at 42 °C with gentle agitation.

Detection and microscopy

The next steps in the detection procedure follow the protocol of Pinkel *et al.* [20] with modifications. Slides were incubated for 5 min in PNM buffer [PN buffer with 5% nonfat dry milk (Carnation)] and then briefly drained. Each slide was layered with 35 μl of 3 μg/ml fluorescein avidin DN (Vector Research Laboratories) in PNM buffer, covered with a parafilm coverslip, and incubated for 20 min at 37 °C. Slides were washed with buffer 3 times for 5 min each at 42 °C. Slides were incubated in 5% normal goat serum in PN buffer, 35 μl per slide, for 5 min at room temperature and then drained. To each, 35 μl of 5 μg/ml biotinylated goat anti-avidin antibody (Vector Research Laboratories) in PN buffer was applied and incubated 20 min at room temperature. Slides were washed as above, and a final layer of fluorescein avidin DN was applied. After washing, slides were mounted in 0.4 μg/ml propidium iodide in an anti-fade solution [11].

Color slides of metaphase plates were taken using a Reichert-Jung Polyvar microscope equipped with a Reichert Plan Apo oil immersion objective (100 ×, 1.32 numerical aperture) using Kodak Ektachrome 400 slide film with exposure times of 120 to 150 s. A B1 epifluorescent module with the following filters was used for simultaneous visualization of propidium iodide-counterstained chromosomes and fluorescein signal: excitation, 450 to 495 nm; dichroic mirror, DS 510 nm; emission, LP 520 nm. A B4 module was used for visualization of the fluorescein signal alone and has the following filters: excitation, 475 to 495 nm; dichroic mirror, DS 510 nm; emission, BP 520 to 560 nm. A G1 module allowed visualization of propidium iodide stained chromosomes without the fluorescein signal: excitation, 546 nm; dichroic mirror, DS 580 nm; emission, LP 590 nm.

Results

Chromosome spreading for in situ *hybridization*

The overall approach for preparation of metaphase chromosomes of sufficient quality for fluorescent *in situ* hybridization involved three major steps including arrest of cells at mitotic metaphase by chemical pretreatment, digestion of cell walls to obtain a relatively clean population of protoplasts, and spreading the chromosomes by lysing protoplasts directly on the slides. The chromosomes were detected by fluorescent dyes such as propidium iodide. *In situ* hybridization was performed using a biotin-labeled soybean ribosomal DNA (rDNA) probe and the regions of homology were visualized using an avidin-fluorescein conjugate similar to procedures for human chromosomes [15, 20].

Metaphase chromosomes free of cellular debris were obtained following modifications of existing methods used previously for *Zea mays* [23], *Sinapis alba* [6], *Crepis capillaris* [1], *Apium graveolens* and *Brassica carinata* [18], and *Triticum monococcum* and *Papaver somniferum* [9]. In general, cellular debris must be removed by enzymatic digestion of the mitotic tissue and numerous washes of the protoplasts before they are spread directly on slides. Initial attempts with

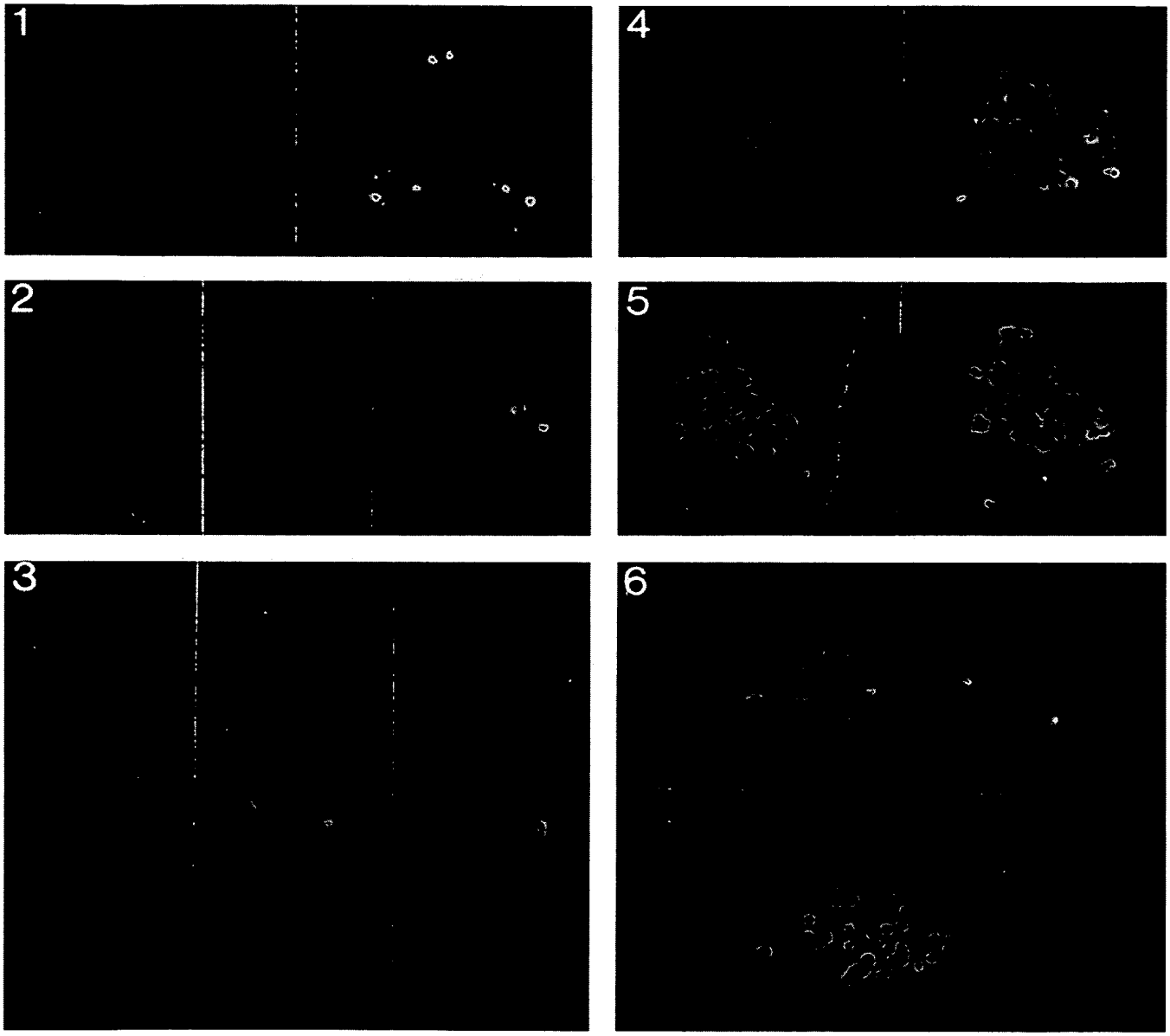

Fig. 1. Fluorescent detection of rDNA within interphase or clumped metaphase chromosomes from soybean root tips. The nick-translated and poly(T)-tailed pSR1.2B3 probe was used. Left, propidium iodide staining of total DNA; right, two very strong yellow fluorescent signals are observed in each of the three cells the B4 filter that detects only the fluorescein label. Magnification × 1200.

Fig. 2. Detection of rDNA genes on soybean metaphase chromosomes. Bio-11-dUTP was incorporated into the pSR1.2B3 probe using nick translation without subsequent poly(T)-tailing. Chromosomes are counterstained with 0.25 μg/ml propidium iodide. Left panel, detection of propidium iodide staining with the G1 filter; middle, bluish fluorescence of the fluorescein signal only using the B4 filter that blocks propidium emission; right, yellow fluoresence of the fluorescein signal on the chromosomes using the B1 filter combination. Magnification × 1200.

soybean yielded slides relatively free of cellular debris but resulted in a high proportion of chromosomes in a 'clumped' arrangement as shown in Fig. 1. Although *in situ* hybridization can be performed on these clumps yielding two distinct fluorescent signals with the rDNA probe (Fig. 1, right), the metaphase chromosomes in this arrangement offer no advantages over interphase cells.

Failure of the soybean chromosomes to spread could result from the pretreatment used for arresting cells, the fixative, or the way slides are prepared. Initially, we used colchicine pretreatment, Farmer's fixative (3 parts ethanol, 1 part acetic acid), and room temperature spreading. We then explored alternatives in an attempt to obtain a higher proportion of well spread chromosomes. Other pretreatment agents used to arrest metaphase cells are para-dichlorobenzene, α-monobromonaphthalene, or 8-hydroxyquinoline. As detailed in Materials and methods, the most promising results were obtained with 8-hydroxyquinoline followed by tissue fixation in methanol-acetic acidchloroform (3 : 1 : 1). Humidity and cold slides sometimes aid chromosome spreading. Best results with soybean chromosomes were obtained when methanol cleaned slides were stored at − 70 °C for several hours before spreading and the prepared cell suspension was dropped immediately onto frosted slides taken from the freezer. Using this combination of pretreatment, fixative, and slide preparation, 50 to 75% of the soybean metaphase cells exhibit 40 well spread chromosomes that allowed direct detection of the *in situ* signal to individual chromosomes as shown in Figs. 2–6.

Comparison of probe labeling methods for in situ hybridization

Nick translation was used to incorporate the bio-11-dUTP into total plasmid DNA and the size of fragments are adjusted to approximately 300–1000 nucleotides. The nick-translated fragments were used directly as a probe or were further labeled by addition of poly(T) tracts to the fragment ends with terminal deoxynucleotide transferase and hybridization to a poly(dA) molecule containing incorporated biotins (BioBridge). Thus, additional biotin residues should increase the amount of label available for detection. This appeared to be the case from comparing the intensity of the nick-translated probe to that of the nick-translated and tailed probe as judged by alkaline gel electrophoresis and detection on gel blots using avidin-alkaline phosphatase (data not shown).

Figures 2 and 3 compare the results of *in situ* hybridization with the two labeling protocols. The rDNA probe was nick-translated with bio-11-dUTP (Fig. 2) or was nick-translated and also poly(T)-tailed (Fig. 3) to incorporate more biotin residues before use as a probe. In each case, two distinct yellow fluorescent signals are visible on separate metaphase chromosomes. However, when signal intensities are compared, it can be seen that signal from the probe that has also been

◀ *Fig. 3.* Enhancement of *in situ* hybridization signal using additional biotin incorporation. Bio-11-dUTP was incorporated into the rDNA probe by nick translation followed by subsequent poly(T)-tailing and hybridization with a poly(dA) : biotin molecule as described in Materials and methods. Left panel, propidium iodide staining of chromosomes; middle, fluorescein signal only; right, simultaneous visualization of the fluorescein and propidium stains.

Figs. 4 and 5. Detection of rDNA sequences on soybean metaphase chromosomes from plants trisomic for chromosome 13. The rDNA probe was labeled by both nick translation and poly(T)-tailing. Left, propidium iodide staining showing 41 chromosomes; right, simultaneous visualization of chromosomes and the fluorescein signal. Three intense yellow fluorescent fluorescein signals are distinguishable in each figure. Magnification × 1200.

Fig. 6. In situ hybridization to a trisomic plant using a nick-translated rDNA probe without subsequent poly(T)-tailing. Top, fluorescein signal on three of the metaphase chromosomes as compared to the propidium iodide stained chromosomes (bottom). Magnification × 1200.

poly(T)-tailed is several-fold more intense than the signal with a probe that is only nick-translated. This is especially evident when the figures that show the fluorescein signals alone are compared (Figs. 2 and 3, middle panels in which the fluorescein is bluish green using this excitation and barrier filter combination). In more than 95% of the chromosome spreads examined, it was apparent that there were two signals for the rDNA probe that were definitely located on individual chromosomes as opposed to background signal.

Analysis of trisomic plants by in situ *hybridization*

A primary trisomic line for the satellited chromosome 13 that associates with the nucleolus [24, 28, 29] was examined. Using the 18S rDNA probe, three very strong fluorescent signals were routinely visualized on individual metaphase chromosomes from plants trisomic for the satellited chromosome as shown in several independent experiments (Figs. 4–6). Unambiguous assignment of the signal was aided by use of filter combinations that allow visualization of the same chromosome spread with either the propidium iodide alone or with the fluorescein signal superimposed on the propidium iodide-stained chromosomes. For example, two adjacent chromosomes in Fig. 5 display signal along with a third separate one. Use of biotinylated goat anti-avidin antibody to amplify the signal generally leads to a higher amount of background fluorescent spots and this step can be omitted for a cleaner preparation. Again, the probes prepared using nick translation and poly(T) additions (Figs. 4 and 5) gave a more intense signal than those with nick translation alone (Fig. 6).

Discussion

The application of nonradioactive *in situ* hybridization has facilitated the mapping of ribosomal RNA genes in several plant genomes [17, 21, 32]. Other repeated DNA sequences have been used as cytological markers in conjunction with nonradioactive *in situ* hybridization in wheat [22] and a low-copy DNA sequence in rye coding for the endosperm storage protein gene, *Sec-1*, has been localized to chromosome 1RS [7]. With few exceptions, *in situ* hybridizations to plant chromosomes have utilized either tritium or nonradioactive, enzymatic detection using biotin-labeled probes. In contrast, fluorescent detection is currently used extensively in human chromosome analysis. Our results and those of Maluszynska and Schweizer [17] demonstrate the feasibility of employing fluorescent detection methods with plant chromosomes using probes to repetitive DNA.

In most of the plant species cited above, the metaphase chromosomes can be karyotyped by size or differential staining. In soybean, however, the 40 metaphase chromosomes are very small. They cannot be karyotyped by size and do not produce significant banding patterns for identification [12, 25]. In order to identify the individual chromosomes, meiotic cells must be employed and the first karyotype of soybean pachytene chromosomes was recently reported [27]. Each individual chromosome was identified by length and its heterochromatic and euchromatic regions. A considerably large segment of the chromosome 13 short arm was associated with the nucleolus in pachytene and often appeared attached but the satellited nucleolar organizing region cannot be recognized in contracted somatic metaphase chromosomes. The 18S and 25S rRNA genes are on the chromosomes that associate with the nucleolus in most organisms. Four primary trisomics of soybean have been identified by pachytene analysis and include chromosomes 1, 4, 5, and 13 [28]. We observed *in situ* hybridization signals on three individual chromosomes of plants trisomic for chromosome 13 (Figs. 4–6). The intense signal from the rDNA probe appears to cover much of the chromosome so that the individual chromatid halves of the mitotic metaphase chromosomes are not distinguished. If the 18S rDNA genes were present on chromosomes other than chromosome 13, fluorescent signal should be observed on additional chromosomes.

We consistently observed only two or three hybridization signals in disomic and trisomic spreads, respectively, that had low background fluorescence. Thus, the 18S rDNA genes appear to be only on chromosome 13 as defined by the limits of sensitivity of this *in situ* hybridization procedure. This agrees with the observation that only chromosome 13 is associated with the nucleolus during pachytene. Although soybean is likely an ancient polyploid, it appears that rDNA genes have been lost by mechanisms that lead to diploidization of many sequences during evolution [29].

Skorupsa *et al.* [29] also detected two and three signals respectively in 'squashes' of interphase cells from soybean disomic and trisomic lines using peroxidase detection of a biotin-labeled maize rDNA probe. However, the signal was not visualized directly on the chromosomes because of difficulties in spreading chromosomes that would survive the *in situ* hybridization conditions. In the present report, we have developed reliable methods for spreading the 40 mitotic soybean chromosomes and have used fluorescent labeling to simultaneously stain the chromosomes and detect the hybridization target sequences.

Further refinements in the *in situ* hybridization technique for soybean will be to localize non-repetitive cloned genes to a particular chromosome. Meiotic karyotyping [27] is currently performed with a 'squash' technique without removal of cell walls which may leave too much debris for *in situ* hybridization to be effective with low background signal. Additionally, it is rare to obtain chromosome spreads that display all 20 meiotic chromosomes well separated within a chromosome spread of an individual cell. *In situ* localization using mitotic metaphase cells from aneuploid lines as demonstrated in this report is a potential alternative. In this regard, we are currently examining the progeny of wide species crosses in an effort to generate and identify alien addition and substitution lines.

Improvements in sensitivity must be achieved to allow rapid and reliable detection of short, non-repetitive probes using pachytene or metaphase soybean chromosomes. There are very few reports of low-copy detection in plants to date and most of these have employed a statistical analysis to verify the presence of the label consistently on a particular chromosome. These include detection of the *waxy* gene on 48% of the chromosome 9 maize pachytene spreads that were examined using tritium detection [26]; tritium detection of the parsley chalcone synthase gene [10]; detection of integrated T-DNA in transformed *Crepis capillaris* [1]; and detection of the low-copy *Sec1* storage protein in rye [7]. Alternatively, it has been found that increasing the target size around a single-copy gene will allow consistent chromosome localizations despite dispersed repeats present in human chromosomes. Thus, overlapping cosmid clones or yeast artificial chromosomes containing large (greater than 100 kb) inserts can be used to effectively hybridize more biotin residues to the target region [30, 19]. The dispersed chromosomal repeats do not interfere or can be suppressed with unlabeled chromosomal DNA before hybridization.

We can roughly calculate the target size of the ribosomal probe used in the present studies in order to estimate the size of a contiguous probe that might be needed to effect a signal for a single-copy gene on the soybean metaphase chromosomes with our current technology. The 18S and 25S rDNA gene unit in soybean has a 7.8 kb total cistronic length and is present in about 500 to 800 copies per haploid genome [3, 5]. Assuming 800 copies and 100% hybridization of the 1.05 kb probe to each of the repeat units, then our signal detected a total of 800 kb over a 6200 kb tandem chromosomal region. A 10-fold reduction in signal intensity should be reliably detectable without computer enhancement based on the very intense signal seen in Figs. 2–6. Thus, an 80 kb minimum target area should suffice. It is not unreasonable to obtain 150–300 kb inserts using yeast artificial cloning technology. In summary, several factors including increased clone size, improvements in spreading pachytene chromosomes, and increased sensitivity for low light levels will be needed to refine the fluorescent *in situ* hybridization process in soybean and other plant species carrying small chromosomes.

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References

1. Ambrose PF, Matzke, AJM, Matzke, MA: Localization of *Agrobacterium rhizognenes* T-DNA in plant chromosomes by *in situ* hybridization. EMBO J 5: 2073–2077 (1986).
2. Albertson DG, Fishpool R, Sherrington P, Nacheva E, Milstein C: Sensitive and high resolution *in situ* hybridization to human chromosomes using biotin labelled probes: assignment of the human thymocyte CD1 antigen genes to chromosome 1. EMBO J 7: 2801–2805 (1988).
3. Eckenrode VK, Arnold J, Meagher RB: Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J Mol Evol 21: 259–269 (1985).
4. Fan YS, Davis LM, Shows TB: Mapping small DNA sequences by fluorescence *in situ* hybridization directly on banded metaphase chromosomes. Proc Natl Acad Sci USA 87: 6223–6227 (1990).
5. Friedrich H, Hemleben V, Meagher RB, Key JL: Purification and restriction endonuclease mapping of soybean 18S and 25S ribosomal RNA genes. Planta 146: 467–473 (1979).
6. Geber G, Schweizer D: Cytochemical heterochromatin differentiation in *Sinapis alba* (Cruciferae) using a simple air-drying technique for producing chromosome spreads. Pl Syst Evol 158: 97–106 (1988).
7. Gustafson JP, Butler E, McIntyre CL: Physical mapping of a low-copy DNA sequence in rye (*Secale cereale* L.). Proc Natl Acad Sci USA 87: 1899–1902 (1990).
8. Haase AT, Retzel EF, Staskus KA: Amplification and detection of lentiviral DNA inside cells. Proc Natl Acad Sci USA 87: 4971–4975 (1990).
9. Hadlaczky Gy, Bisztray Gy, Praznovsky T, Dudits D: Mass isolation of plant chromosomes and nuclei. Planta 157: 278–285 (1983).
10. Huang PL, Hahlbrock K, Somssich IE: Detection of a single-copy gene on plant chromosomes by *in situ* hybridization. Mol Gen Genet 211: 143–147 (1988).
11. Johnson GD, Nogueira Araujo GM: A simple method of reducing the fading of immunofluorescence during microscopy. J Immun Meth 43: 349–350 (1981).
12. Ladizinsky G, Newell CA, Hymowitz T: Giemsa staining of soybean chromosomes. J Hered 70: 415–416 (1979).
13. Landegent JE, Jansen in de Wal N, van Ommen G-JB, Baas F, de Vijlder JJM, van Duijn P, van der Ploeg M: Chromosomal localization of a unique gene by non-autoradiographic *in situ* hybridization. Nature 317: 175–177 (1985).
14. Lawrence JB, Marselle LM, Byron KS, Johnson CV, Sullivan JL, Singer RH: Subcellular localization of low-abundance human immunodeficiency virus nucleic acid sequences visualized by fluorescence *in situ* hybridization. Proc Natl Acad Sci USA 87: 5420–5424 (1990).
15. Lawrence JB, Villnave CA, Singer RH: Sensitive, high-resolution chromatin and chromosome mapping *in situ*: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell 52: 51–61 (1988).
16. Lichter P, Tang C-JC, Call K, Hermanson G, Evans GA, Housman D, Ward DC: High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science 247: 64–69 (1990).
17. Maluszynska J, Schweizer D: Ribosomal RNA genes in B chromosomes of *Crepis capillaris* detected by non-radioactive *in situ* hybridization. Heredity 62: 59–65 (1989).
18. Murata M: Staining air dried protoplasts for study of plant chromosomes. Stain Technol 58: 101–106 (1983).
19. Pachnis V, Pevny L, Rothstein R, Costantini F: Transfer of a yeast artificial chromosome carrying human DNA from *Saccharomyces cerevisiae* into mammalian cells. Proc Natl Acad Sci USA 87: 5109–5113 (1990).
20. Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83: 2934–2938 (1986).
21. Price HJ, Stelly DM, McKnight TD, Scheuring CF, Raska D, Michaelson MJ, Bergey D: Molecular cytogenetic mapping of a nucleolar organizer region in cotton. J Hered 81: 365–370 (1990).
22. Rayburn AL, Gill BS: Use of repeated DNA sequences as cytological markers. Amer J Bot 74: 574–580 (1987).
23. Rayburn AL, Gold JR: A procedure for obtaining mitotic chromosomes from maize. Maydica 25: 113–121 (1982).
24. Sadanaga K, Grindeland RL: Aneuploids and chromosome abberrations from irradiated soybeans. Soybean Genet Newsl 6: 43–45 (1979).
25. Sen NK, Vidyabhusan RV: Tetraploid soybean. Euphytica 9: 317–322 (1960).
26. Shen D, Wang Z, Wu M: Gene mapping on maize pachytene chromosomes by *in situ* hybridization. Chromosoma 95: 311–314 (1987).
27. Singh RJ, Hymowitz T: The genomic relationship between *Glycine max* (L.) Merr. and *G. soja* Sieb. and Zucc. as revealed by pachytene chromosome analysis. Theor Appl Genet 76: 705–711 (1988).
28. Singh RJ, Hymowitz T: Identification of four primary

trisomics of soybean by pachytene chromosome analysis. J Hered 82: 75–79. (1991).
29. Skorupsa H, Albertsen MC, Langholz KD, Palmer RG: Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by *in situ* hybridization. Genome 32: 1091–1095 (1989).
30. Trask BJ, Hamlin JL: Early dihydrofolate reductase gene amplification events in CHO cells usually occur on the same chromosome arm as the original locus. Genes Dev 3: 1913–1925 (1989).
31. Viegas-Pequignot E, Dutrillaux B, Magdelenat H, Coppey-Moisan M: Mapping of single-copy DNA sequences on human chromosomes by *in situ* hybridization with biotinylated probes: Enhancement of detection sensitivity by intensified-fluorescence digital-imaging microscopy. Proc Natl Acad Sci USA 86: 582–586 (1989).
32. Visser RGF, Hoekstra R, van der Leij FR, Pijnacker LP, Witholt B, Feenstra WJ: *In situ* hybridization to somatic metaphase chromosomes of potato. Theor Appl Genet 76: 420–424 (1988).

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High-resolution mapping of repetitive DNA by *in situ* hybridization: molecular and chromosomal features of prominent dispersed and discretely localized DNA families from the wild beet species *Beta procumbens*

T. Schmidt and J.S. Heslop-Harrison
Department of Cell Biology, John Innes Centre, Colney, Norwich NR4 7UJ, UK

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Abstract

Members of three prominent DNA families of *Beta procumbens* have been isolated as *Sau*3A repeats. Two families consisting of repeats of about 158 bp and 312 bp are organized as satellite DNAs (*Sau*3A satellites I and II), whereas the third family with a repeat length of 202 bp is interspersed throughout the genome. Multi-colour fluorescence *in situ* hybridization was used for physical mapping of the DNA families, and has shown that these tandemly organized families occur in large heterochromatic and DAPI positive blocks. The *Sau*3A satellite I hybridized exclusively around or near the centromeres of 10, 11 or 12 chromosomes. The *Sau*3A satellite family I showed high intraspecific variability and high-resolution physical mapping was performed on pachytene chromosomes using differentially labelled repeats. The physical order of satellite subfamily arrays along a chromosome was visualized and provided evidence that large arrays of plant satellite repeats are not contiguous and consist of distinct subfamily domains. Re-hybridization of a heterologous rRNA probe to mitotic metaphase chromosomes revealed that the 18S-5.8S-25S rRNA genes are located at subterminal position on one chromosome pair missing repeat clusters of the *Sau*3A satellite family I. It is known that arrays of *Sau*3A satellite I repeats are tightly linked to a nematode (*Heterodera schachtii*) resistance gene and our results show that the gene might be located close to the centromere. Large arrays of the *Sau*3A satellite II were found in centromeric regions of 16 chromosomes and, in addition, a considerable interspersion of repeats over all chromosomes was observed. The family of interspersed 202 bp repeats is uniformly distributed over all chromosomes and largely excluded from the rRNA gene cluster but shows local amplification in some regions. Southern hybridization has shown that all three families are specific for genomes of the section *Procumbentes* of the genus *Beta*.

The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z22862 (pTS2), Z50807 (pTS1), Z50809 (pTS5), Z50810 (pTS6), Z50811 (pTS23), Z50812 (pTS100), Z50808 (pTS4.1) and Z22863 (pTS3).

Introduction

Most differences in composition and complexity of higher plant genomes can be defined by repetitive DNA, and all plant species examined so far contain numerous DNA sequences with repeating units varying widely in length and complexity. Two main types of repetitive DNA are distinguishable by their genomic organization [for review see 7 and 35]. Dispersed repetitive DNA is a heterogeneous class of sequences with repeats interspersed throughout the genome and scattered over all or many chromosomes of the complement [5, 13, 21, 40, 45]. However, it has been shown that the interspersion pattern of some repeats is often not random and that, in particular, mobile sequences like retrotransposons occur clustered in distinct regions of the genome [53].

Another class of repetitive sequences, known as satellite DNA [57], is organized in tandem arrays, often amplified up to 10^5 to 10^6 copies/1C and can account for several percent of the nuclear DNA. Arrays of satellite DNA are predominantly found in subtelomeric or centromeric heterochromatin blocks on single, multiple or all chromosomes as shown by *in situ* hybridization [22, 24, 31, 39, 63].

Although conservation of many tandem repeat families across species borders has been observed, their diversification in nucleotide sequence, abundance and chromosomal localization over different evolutionary time scales often results in co-existence of closely related, diverged satellite DNA subsets or subfamilies within a genome [30]. In *Drosophila melanogaster* and human it has been shown that long arrays of tandem repeats are not contiguous and homogenized, and diverged satellite DNA subsets reside either in distinct domains of an individual chromosome or on separate chromosomes [8, 37, 64]. In contrast, the long-range analysis of the TGRI satellite family in tomato lead to the suggestion that blocks of satellite sequences are homogenous and uninterrupted, and located at individual chromosomal sites [12, 34].

Some families of tandemly repeated sequences undergo more rapid structural changes during speciation, and considerable divergence resulting in genus- or species-specific satellite sequences have been observed between closely related genera and species [17, 19, 51]. The investigation of satellite DNA conservation, divergence and reorganization can support taxonomic and phylogenetic studies [15, 18, 23, 59]. While knowledge about the evolutionary relationships of diverged satellite DNA families leads to insights and understanding of genome organization and evolution, some of the analyzed satellite repeats have also proved to be useful molecular markers in plant breeding, and the selection of interspecies hybrids using genome-specific satellite repeats has been described for many crops [2, 55, 56, 61, 65, 66].

The genus *Beta* is taxonomically divided into the sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. Sugar beet, fodder beet, beet root and mangold are cultivated forms of the species *Beta vulgaris* L. ($2n = 18$) in the section *Beta*. Their genomes contain some 60% repetitive DNA sequences [11] and have a haploid genome size of about 750 Mb [4]. Despite the small size of the chromosomes, both mitotic and meiotic karyotypes have been made and enabled chromosomal analyses of *Beta* species [9]. Wild beets of the sections *Corollinae* and *Procumbentes* provide valuable genetic resources for breeders because of their potential to serve as donors of agronomically valuable genes. Genes giving resistance or tolerance to the beet cyst nematode (*Heterodera schachtii* Schm.) have been found in wild beet species of the section *Procumbentes*, and intersection crosses with *B. vulgaris* established nematode-resistant lines including monosomic additions ($2n = 19$), fragment additions or diploid translocation [6, 20, 28, 38, 42, 49]. A set of repetitive DNA sequences specific for the genome of *B. procumbens* has been isolated and proved to be useful markers for the selection of resistant breeding lines of *B. vulgaris* in rapid screening approaches [52, 25]. Arrays of one repetitive sequence have been found physically close to the nematode resistance locus as shown by analyses of YAC and lambda clones spanning the intro-

gressed wild beet chromosome segment in *B. vulgaris* [33, 32, 46].

Nothing is known about the chromosomal distribution and organization of genome-specific, highly repetitive sequences within the genome of *B. procumbens*. Here we describe the molecular cytogenetics of prominent repetitive DNA families from *B. procumbens*. We have analysed the molecular structure, genomic organization and interspecies distribution of two non-homologous satellite repeats and one family of highly repeated, interspersed DNA sequences. Multi-colour fluorescence *in situ* hybridization was used for physical mapping of repetitive DNA families and genes coding for the 18S-5.8S-25S rRNA on mitotic metaphase chromosomes of *B. procumbens*, while the analysis of extended meiotic chromosomes at pachytene enabled the localization of satellite subsets in discrete domains.

Material and methods

Plant material

Wild beet species used in this study were kindly provided by Dr L. Frese (Institut für Pflanzenbau, FAL Braunschweig, FRG) and Dr B. Fordloyd (University of Birmingham). *Beta vulgaris* cv. Rosamona and *Spinacea oleracea* are commercially available cultivars. Plants were grown under green house conditions. *Chenopodium bonus-henricus* was collected in the Botanical Garden of the University Halle (FRG). All genera investigated belong to the *Chenopodiaceae* family.

DNA isolation, cloning and Southern hybridization

Genomic DNA from *B. procumbens* was isolated from fresh leaf material, digested with *Sau*3A and cloned into pUC18. Recombinant clones containing repetitive DNA sequences were selected by colony or dot blot hybridization with genomic DNA [52]. To isolate and clone a full length repeat, the following primers were designed from a partial monomer of the *Sau*3A satellite family II and used for the polymerase chain reaction: 5'-CGAAAACGGTAAATCGG-3' and 5'-GTGGACTATTACTAACGCC-3'. PCR was carried out for 35 cycles, each comprising of a 1 min at 92 °C, 1 min at 54 °C and 1 min at 72 °C. The PCR product, consisting of the basic repeats and multimers thereof, was cloned into pUC18. For Southern blot analyses genomic DNA from wild and cultivated beet species was digested, electrophoresed on 1.2% agarose gels and transferred onto nylon membranes using standard techniques [48]. After labelling with [α-^{32}P]dATP by random priming, clones were hybridized to Southern blots at 62 °C overnight and washed in 2 × SSC/0.1% SDS at 62 °C.

Fluorescence in situ *hybridization*

Young flower buds from *B. procumbens*, treated in 2 mM 8-hydroxyquinoline for 2 h and fixed in methanol/acetic acid (3:1), were used as material for *in situ* hybridization. Chromosomes were prepared after 2.5 h digestion at 37 °C in enzyme solution consisting of 2% cellulase (Calbiochem) and 20% pectinase in 10 mM citric acid/sodium citrate pH 4.6. *In situ* hybridization was performed as described [54] with 79% stringency according to Meinkoth and Wahl [41]. Probes were labelled with digoxigenin-11-dUTP or biotin-11-dUTP using PCR and detected with FITC conjugated anti-digoxigenin antibody (green images) and streptavidin-Cy3 conjugate (red images), respectively. Slides were counterstained with DAPI (4',6-diamino-2-phenylindole) and mounted in antifade solution. For reprobing, the cover slip was removed and slides were washed in 2 × SSC, dehydrated in an ethanol series and re-hybridized with a differently labelled probe. Slides were examined with a Leica epifluorescence microscope with filters sets A, I2/3 and N2.1. Microphotographs were taken on colour print film, digitized to photo CD and printed using Adobe Photoshop after contrast optimization of the whole image.

Results

Molecular structure of major repeat families of B. procumbens

Three families of repeated DNA sequences were isolated from the genome of *B. procumbens* by random cloning of *Sau*3A fragments followed by screening of the resulting plasmid libraries for highly repetitive clones.

Members of one sequence family, referred to as the *Sau*3A satellite I or 158 bp repeat family, are represented by the inserts of pTS1, pTS2, pTS5, pTS6 and pTS100 and shown in Fig. 1A. The monomers are 158–159 bp long and contain 68–71% adenine and thymidine residues. Internal *Sau*3A recognition sites, formed by single base pair mutations, released partial repeat units in pTS1 and pTS23, which are 94 bp and 130 bp long, respectively, but belong to the same family of repeats. More than 24% sequence divergence between monomer units was detected. Although many of the observed mutations show no apparent patterns, some occur in more than one repeat suggesting that these alterations have been fixed in subsets of the satellite family. The occurrence of conserved nucleotides allowed the discrimination of repeat variants, with the monomer pTS6 showing the highest number of nucleotide alterations. In addition to single nucleotide mutations, a deletion of 7 bp from nucleotide 102 to 109, was found.

Southern hybridization to genomic DNA blots was performed to investigate the genomic organization of the 158 bp repeat family (Fig. 2A). A ladder pattern typical for a satellite DNA genome organization was detected in *Sau*3A and *Dra*I digests after probing with the labelled repeat pTS5. Both restriction enzymes have recognition sites within most analysed repeats, and the strongest hybridization signals in these digests corresponded to monomers. However, digestion with *Sau*3A revealed also a population of fragments of approximately half the monomer size visible as a less intensive hybridization signal below the monomers. This observation indicates that in a subset additional recognition sites have emerged and is consistent with the observed sequence divergence and detection of internal *Sau*3A sites in pTS1 and pTS23. Hybridization to DNA digested with *Bam*HI, *Bgl*II and *Hin*dIII resulted in intense signals greater than 20 kb demonstrating the absence of these sites within tandem arrays, although some smear, in particular in *Bgl*II digests, was observed. Taken together, these results reflect that sequences of this prominent DNA family of *B. procumbens* are organized in large clusters of long tandem arrays. Studies of the methylation, performed by Southern hybridization using the isochizomeric enzyme pairs *Sau*3A/*Nde*II and *Hpa*II/*Msp*I differing in sensitivity to 5-methylation of cytosine [43], revealed that GATC sites show some methylation of the cytosine, whereas in most CCGG sites both cytosine residues are methylated (Fig. 3). Although no CCGG sites were found in the clones analysed, those sites are likely to be present in other members of the family, since there are several positions within the repeats where a single base pair mutation can result in a *Hpa*II/*Msp*I recognition site.

In contrast, a significant higher methylation of the internal cytosine of CCGG sites and a similar proportion of methylated cytosine in GATC sites is characteristic for members of a second large family of *B. procumbens* repeats (Fig. 3b).

To isolate a full length monomer of the second sequence family, PCR was performed with primers designed from a partial repeat [52]. Amplification of genomic DNA resulted in products consisting of a basic repeat and multimers thereof. Purified PCR products were digested with *Sau*3A and cloned. The selected clone pTS4.1 contains a monomer of 312 bp and an A + T content of 49% (Fig. 1b). This sequence represents a large fraction of the wild beet genome and is called

Fig. 1. Nucleotide sequence of the *Sau*3A satellite DNA I (A) and II (B) and the family of dispersed 202 bp repeats from *B. procumbens* (C). For comparison, the repeat pRK643 [27] and the consensus sequences of the Sat121.1 and Sat121.2 [46] were included in A. Identical nucleotides are shown by dots. Gaps introduced for optimal alignment are shown by dashes.

A Sau3A satellite family I

```
                10          20          30          40          50
pTS2      GATCCAAGGC -TTCATATGC TTTAAATATA TCTAATACCC ATTCAAGGAG
pTS1                                    G. ..-.G....T .........C
pTS5      .......... -..AG..... ....C..... C.........T ...A......
pTS6      .........G A..T....T. .......... .........T ..........
pTS23
pTS100    .........G A.........A ......C... .......T.T ..........
pRK643    ....TG...G C.......... ....C..... C.......A.T ...A......
Sat121.1  .........G C.......... ....C..... C.........T ...A.....A
Sat121.2  .........G C.......... .......... .........T ..........

                60          70          80          90          100
pTS2      ACAAAAAATA TTTGGTAATT AAGCACAAAA AA-TGACTTA AAAAG-TGTG
pTS1      T......-.. ...T...... .TTA....C. ..A.T.T..G ...-.T...T
pTS5      T......-C. A.A....... .......TC. ..-...T..G ...-.G...T
pTS6      T......-.. ..C....... ........C. ..-.T.T..G ..T..-..AC
pTS23                      G..C .TT.CTG.C. ..A..AT..G ...-.G.T.T
pTS100    T......-.. G.......... ........C. ......T..T ...-.G...T
pRK643    T......-C. A.A....... .......TCC ..-...T..G ...-.G...T
Sat121.1  TA.....-CA A.A....... .......TC. ..-...T..G ...-.G...T
Sat121.2  T......-.A .......... .T......C. ..A.A.T..G ...-.G...T

                110         120         130         140         150
pTS2      --CATACCCC ACACA-TCCC CTAATAGACT ATGACGGTTTT ACCCTTTGT
pTS1      --.....A.. ...A.-..G. ....G..... ..........A ........A
pTS5      T-.....A.A ...A.-..T. G...G.T... ....TA..... .A......A
pTS6      AC...----- --.A.-..G. ....G...TA ......A.... ........A
pTS23     --.....A.. .T.A.-..A. ....G..... T.......... ........A
pTS100    --..A..A.- ...A.-..G. ....G...A. ..A...A.... ........A
pRK643    --T....ATA T..A.-..T. G...G..... ....TA...-- ..---...A
Sat121.1  --.....A.. ...AAA..T. G...G..... ....TA..... .A......A
Sat121.2  --.....A.. ...AA-..G. ....G..... .......... ........A

              160
pTS2      TTTAAAAT AAGTTT
pTS1      ......T. G.....
pTS5      ...G.... G.....
pTS6      ........ GC....
pTS23     ...G.... ......
pTS100    .A.G.... ......
pRK643    -..G..-. G....-
Sat121.1  ...G.... G.....
Sat121.2  ...G.... A.....
```

B Sau3A satellite family II (pTS4.1)

```
         10          20          30          40          50
GATCATGTCC AAAAATATTT TAGGGCCTCC TTGGGCCAAA TGACGCCCTT

         60          70          80          90          100
TGGAACCTTA ACATGCCGAA AATCGGGTAC CGGATACCTT CCAATCCCAC

         110         120         130         140         150
CTCCGTAATT CCGACCAATA TCGATAACCG ACTCGGGAGG CCATTTAGAG

         160         170         180         190         200
ACTCTTTTGG GGCTTGAAGC CCCCCAAAAT TTACAGAGAC CCTATGGGGA

         210         220         230         240         250
CCCCCGGGAA GGCACATGTG AAAAAAAATT ACCCTGCCCA AAAATGTTCC

         260         270         280         290         300
GATTTACCCG TTTTTGGTGG ACTATTACTA ACGCCCCGGC CACGACCCAG

         310
GGTCCGGAGT TG
```

C Sau3A sequence family III (pTS3)

```
         10          20          30          40          50
GATCAAAAGG AAGGATTGGA GATGAATTTT TGATGGCCTT AGGTTAGTCA

         60          70          80          90          100
ATAGGTGACT TCACATGAGA ATAGTGTTAG GCTTCTAGGA CCTTAATCGA

         110         120         130         140         150
AGCTATGAGG TGGAATATGA TCGATATTTT ACCTAGAGTA GGAAATTGGT

         160         170         180         190         200
TTTAAGTCCC TAATTAGCGC GGGTAATTAG GAACAAGAGT GCTATCCCTT

AT
```

Fig. 2. Genomic organization of prominent repeat families of *B. procumbens*. DNA from *B. procumbens* was digested with *Bam*HI (1), *Bgl*II (2), *Sau*3A (3), *Dra*I (4) and *Hin*dIII (5), electrophoresed and blotted. Identical filters were hybridized with the probes pTS5 (A), pTS4.1 (B) and pTS3 (C). Size marker from top 23.1 kb, 9.4 kb, 6.5 kb, 4.4 kb, 2.3 kb and 2.0 bp.

Fig. 3. Methylation of *B. procumbens* satellite DNA sequences. Genomic DNA was digested with *Sau*3A (A), *Nde*II (2), *Hpa*II (3) and *Msp*I (4), electrophoresed and blotted. Filters were hybridized with probes pTS5 of repeat family I (A) and pTS4.1 of repeat family II (B). Size markers from top: 23.1 kb, 9.4 kb, 6.5 kb, 4.4 kb, 2.3 kb and 2.0 kb.

*Sau*3A satellite II or 312 bp repeat family. The homology to the *Sau*3A satellite family I from *B. procumbens* is confined to the 13 bp motif 5′-CAAAAAATATTTT-3′ that was found in both pTS4.1 and pTS1. Internal subrepeats were not observed, and short sequence repetitions mostly consisted of mononucleotide stretches. The molecular arrangement of the 312 bp repeats was studied by genomic Southern hybridization and revealed, by the ladder-like hybridization pattern with increasing fragments in *Sau*3A digests and indicative in *Dra*I and *Bam*HI digests, that this sequence is organized in tandem arrays and hence is a second *Sau*3A satellite of the *B. procumbens* genome (Fig. 2B). However, compared to the *Sau*3A satellite family I, the ladder pattern is less pronounced even after longer exposure implying an additional interspersion of the repeats into many regions of the genome. This finding is supported by the observed smear over a wide range of middle and high molecular weight fragments visible after hybridization to *Bam*HI, *Bgl*II, *Dra*I- and *Hin*dIII-digested genomic DNA.

A third major sequence family, represented by pTS3, was isolated by screening of a *B. procumbens* plasmid library. The clone pTS3 harbours a repetitive *Sau*3A fragment of 202 bp with 61% adenine and thymidine residues and has no homology to the 158 bp and 312 bp satellite DNA family (Fig. 1C). In contrast to both *Sau*3A satellite DNAs with repeats arranged in tandem arrays, this sequence family shows a different genomic organization. The insert of pTS3 is a highly repetitive component of the *B. procumbens* genome and interspersed with many different genomic sequences, displayed by the complex hybridization patterns in *Dra*I and *Hin*dIII digests (Fig. 2C). However, the *Sau*3A repeat III is also part of a larger repetition unit detected as a strongly hybridizing 1.5 kb *Bgl*II fragment of moderate copy number. It is likely that these 1.5 kb fragments represent a higher order unit which is amplified and tandemly organized in some regions of the *B. procumbens* genome. Despite the similarity to the complex Southern hybridization pattern of retrotransposon-like sequences no homology to those elements was found.

Computer analysis of the structure of all three sequence families showed small inverted or direct

repeats, but none of them was significant and noteworthy.

Localization of repeat families on chromosomes of B. procumbens

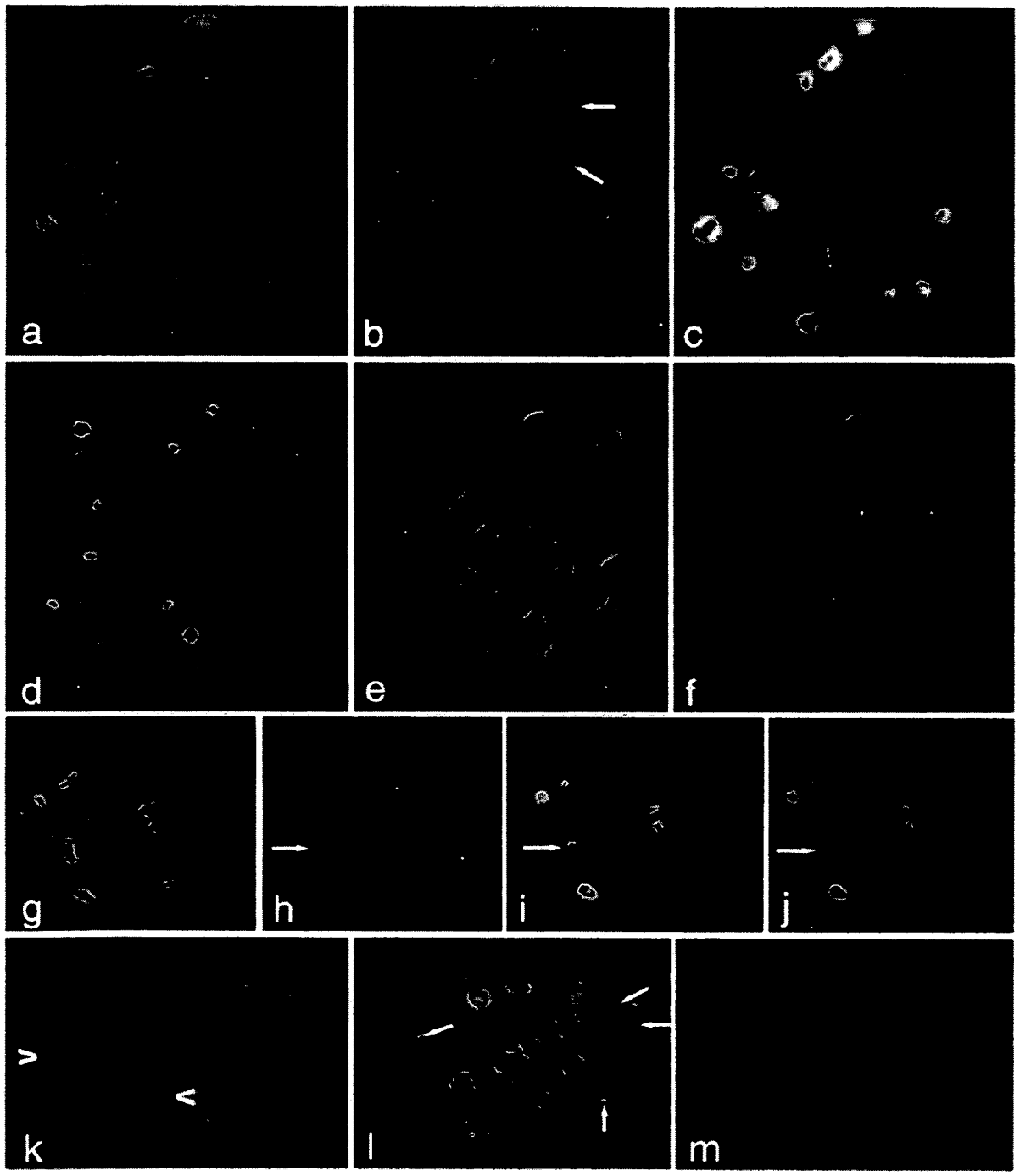
The chromosomes for the physical mapping by fluorescence *in situ* hybridization were prepared from flower buds of *B. procumbens*. Fig. 4a–m shows micrographs after fluorescence *in situ* hybridization and counterstaining with the DNA-specific fluorochrome DAPI. Under UV excitation, the DAPI stained submetacentric to subtelocentric chromosomes of *B. procumbens* showed large heterochromatic chromomers around the centromeric constriction.

Members of the *Sau*3A satellite family I, consisting of 158 bp repeats, reside in DAPI stained chromosomes of 10 to 12 chromosomes. Hybridization with clone pTS5 revealed a distribution of the *Sau*3A satellite I near or around the centromeres (Fig. 4a–d, k, m). One pair of very strong hybridization signals was localized within the brightly DAPI-stained region of one chromosome pair with subterminal centromeric constriction. These major sites represent very large blocks of tandem arrays of the 158 bp *Sau*3A repeat. A pair of minor sites was found on two subtelocentric chromosomes (Fig. 4b). The hybridization signal was weak but reproducible and found in all metaphases. In contrast, the number of the remaining sites hybridizing with middle to strong signal varied between six and eight depending on the plant investigated. Slides were re-hybridized with the heterologous probe pTa71 which contains the 18S-5.8S-25S rRNA genes and intergenic spacer sequences from *Triticum aestivum* [14]. In *B. procumbens* the genes for the 18S-5.8S-25S rRNA were mapped at a subterminal position on the short arm of one chromosome pair (Fig. 4c, d). No hybridization of pTS5 was found on the NOR chromosomes and, hence this pair of chromosomes does not carry repeats of the *Sau*3A satellite family in detectable copy number.

The sequence analysis of six cloned monomers revealed considerable divergence between repeats of the *Sau*3A satellite family I. The repeats pTS5 and pTS6 share only 75.2% identity, and specific nucleotide alterations and an internal deletion allow a clear differentiation. Multi-colour *in situ* hybridization with pTS5 and pTS6, labelled with different fluorochromes, was carried out to examine the chromosomal distribution of the *Sau*3A satellite subfamilies. Mitotic chromosomes of genus *Beta* are relatively small, and therefore more extended pachytene (meiotic prophase) nuclei were prepared and used for *in situ* hybridization. The hybridization was performed at 76% stringency, post-hybridization washes were done at 79% stringency and hence were above the internal homology between pTS5 and pTS6. Both satellite subfamilies hybridized to similar chromosomal locations as detected by red signals of pTS5 and green signals of pTS6. The hybridization sites correspond to brightly stained heterochromatin blocks visible as blue DAPI fluorescence (Fig. 4g).

Under the stringent hybridization conditions choosen here, the two subfamilies are not interspersed but organized in distinct domains. One large array of satellite repeats (arrowed in Fig. 4h, j) comprises at least three repeat domains mapped in the order pTS6-pTS5-pTS6 as visualized by multi-colour *in situ* hybridization and image superimposition (Fig. 4i). The extended pachytene chromosomes enabled the detection of a long tandem array consisting of repeats of the pTS5 subfamily and showing red fluorescence. This pTS5 domain is flanked by long stretches of repeats belonging to the diverged pTS6-like subfamily and detected as green signal. The superimposed image showed that all three domains are adjacent to each other.

The *Sau*3A satellite family II, composed of 312 bp repeats, was physically mapped with the probe pTS4.1. The strongest hybridization signals were found in heterochromatic, DAPI-positive regions of 16 *B. procumbens* chromosomes (Fig. 4e, f). The size of the hybridizing regions varied over a relatively wide range, caused by different numbers of repeats on individual chromosomal sites. By double-target *in situ* hybridization using the differently labelled probes


a
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l
m

pTS4.1 and pTS5, the two strongest hybridization sites of the *Sau*3A repeat families I and II were co-localized on one chromosome pair (not shown). However, despite an organization in tandem arrays there is also considerable dispersion of the 312 bp repeat throughout the genome, depicted by weak hybridization of pTS4.1 along all 18 chromosomes of *B. procumbens* (Fig. 4f).

The chromosomal localization of the third prominent *B. procumbens* repeat was investigated by hybridization of the digoxigenin-labelled probe pTS3 to mitotic metaphase chromosomes. After detection with an FITC-conjugated antibody, uniform hybridization signals along all chromosome arms were observed and showed the dispersed distribution of this sequence class (Fig. 4k, l). However, almost no hybridization signals were discovered on the chromosome arm with the secondary constriction, indicating exclusion of pTS3 from a chromosomal region, which mainly consists of tandem arrays of the 18S-5.8S-25S rRNA genes. In addition to the interspersed distribution, discrete clusters were detected at single intercalary domains on four chromosomes, presumably displaying the local amplification of pTS3-like sequences (arrowed in Fig. 4l). By double-labelling *in situ* hybridization experiments it was shown that the intercalary amplification and clustering of pTS3 occurs on chromosomes which lack the 158 bp repeats of the *Sau*3A satellite family I (Fig. 4l, m).

Distribution of the three Sau*3A families within species of the genus* Beta

Southern hybridization experiments were performed to investigate whether related genomes in the other three sections of the genus and species in the *Chenopodiaceae* contain sequences with homology to the major *B. procumbens* repeat classes. Hybridization with repeats of the three DNA families from *B. procumbens* revealed a very similar picture (Fig. 5A–C). All three DNA families show identical hybridization patterns in the genome of *B. patellaris* indicating that no rearrangement occurred. Both *B. procumbens* and *B. patellaris* belong to the section *Procumbentes*, and the similar genomic organization of all three repetitive sequences is in agreement with their close taxonomic relationship. The *B. procumbens* repeats were also detected in *B. nana*, although the very weak hybridization signal indicates a high divergence of the hybridized sequences which, however, does not exclude a homology in short internal domains. The 158 bp repeats of the *Sau*3A family I from *B. procumbens* hybridized with *B. nana* fragments of high molecular weight

←

Fig. 4. In situ hybridization showing the localization of repetitive DNA sequences and 18S-5.8S-25S rRNA genes on chromosomes prepared from flower buds of *B. procumbens*. a. Metaphase chromosomes stained with DAPI (blue fluorescence). b. The same metaphase chromosomes after *in situ* hybridization with the *Sau*3A repeat family I (pTS5). Major sites are visible by bright red fluorescence and arrows point to a pair of minor satellite repeat clusters. c. Re-hybridization of the same metaphase with an rRNA gene probe shows arrays of the 18S-5.8S-25S rRNA genes subterminal on one pair of chromosomes (green fluorescence). d. mitotic prophase stained with DAPI (blue) after double-target *in situ* hybridization with the *Sau*3A repeat I (green signal) and the 18S-5.8S-25S rRNA gene probe (red signal). e. Blue fluorescence of metaphase chromosomes after DAPI staining. f. The same metaphase after hybridization with the *Sau*3A satellite II pTS4.1. Bright red signals at centromeres and red fluorescence of the chromosomes show the organization of *Sau*3A satellite II repeats in major clusters with considerable dispersion along all arms. g–j. High-resolution analysis of *Sau*3A satellite I subfamilies on meiotic pachytene chromosomes. g. DAPI-stained pachytene chromosomes. h. The same chromosome spread after *in situ* hybridization with pTS5 (red signal) showing one array (arrow) flanked by repeats of the pTS6 subfamily (see j). j. Green fluorescence visualizes the distribution of the subfamily represented by pTS6. The arrow is directed to the gap where an array of the diverged pTS5 subfamily resides. i. Overlayed image of h and j. K. DAPI stained metaphase chromosomes. The NOR chromosomes are indicated with open arrowheads. L. *In situ* hybridization with clone pTS3, representing a family of dispersed *Sau*3A repeats. Chromosomal distribution over all chromosomes is visible as yellowgreen fluorescence. Local amplification of pTS3 repeats are indicated by arrows. m. The same metaphase after hybridization with clone pTS5 of the *Sau*3A family I (red signals).

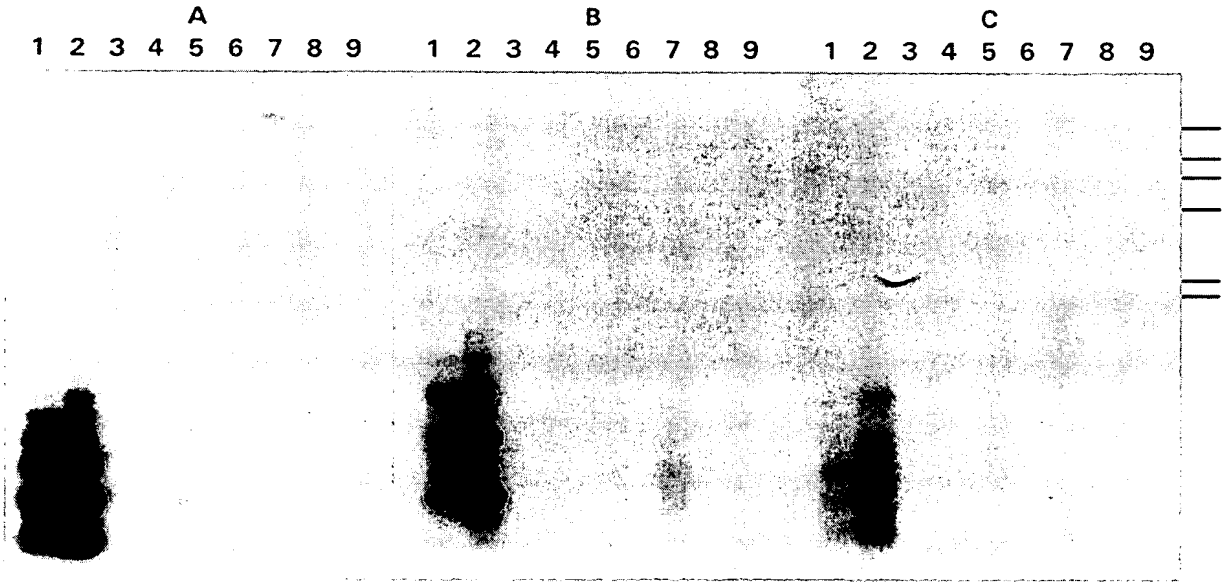

Fig. 5. Genomic distribution of three major repeat families of *B. procumbens* in related *Chenopodiaceae* genomes. *Sau*3A-digested DNA of *B. procumbens* (1), *B. patellaris* (2), *B. vulgaris* ssp. *altissima* (3), *B. vulgaris* ssp. *maritima* (4), *B. lomatogona* (5), *B. corolliflora* (6), *B. nana* (7), *S. oleracea* (8) and *C. bonus-henricus* (9) was electrophoresed and blotted. Filters were hybridized with the probes pTS5 of family I (A), pTS4.1 of family II (B) and pTS3 of family III (C). Size marker from top 23.1 kb, 9.4 kb, 6.5 kb, 4.4 kb, 2.3 kb and 2.0 kb.

which lack *Sau*3A sites and probably have a different genomic organization (Fig. 5A). No hybridization was observed in the remaining species of the genus *Beta*, and in *S. oleracea* and *C. bonus-henricus* indicating that the investigated repetitive DNA families or related motifs are absent or greatly diverged in these genomes.

Discussion

We have investigated the genomic organization, chromosomal localization and interspecies distribution of three *Sau*3A repeat families from *B. procumbens* by Southern and fluorescence *in situ* hybridization (Figs. 2–5).

The three repeat classes account for a considerable fraction of the repeated DNA. The homology of the *Sau*3A satellite families I (pTS1, 2, 5, 6, 100) and II (pTS4.1) is restricted to short stretches only with a maximum of a 13 bp A + T-rich sequence motif, indicating that the *Sau*3A satellite families evolved from different ancestral sequences (Fig. 1A, B). The study of cytosine methylation [16] revealed that sequences of the *Sau*3A family I are less methylated than sequences of the *Sau*3A family II (Fig. 3). That might be explained by the different G + C content which is ca. 20% higher in the *Sau*3A family II (Fig. 1A,B), but might also reflect a difference within the genetic methylation pattern of both families. Methylation of repetitive DNA in plants is variable, but also under genetic control as shown by the investigation of hypomethylation mutants of *A. thaliana* [62] and an undermethylated satellite DNA family in *Pennisetum glaucum* [31].

The *Sau*3A family I consists of monomers of about 158 bp, a typical nucleosome repeat, and resembles a prominent DNA family of *B. procumbens*. First isolated as pTS1 and pTS2 [52], homologous sequences and hence members of the same sequence family were isolated as pRK643 [27], probe 121-3 from *B. patellaris* [47] and

Sat121.1 and Sat121.2 [46] (Fig. 1A). By slot blot hybridizations we calculated a copy number of this satellite DNA family of about 30000 to 40000 in a diploid genome, but due to the observed sequence divergence this value must be considered only as an estimation of the order of magnitude (data not shown). From the strength of hybridization we estimate the number of 312 bp repeats of the *Sau*3A satellite family II to be lower.

Satellite DNA is often found in heterochromatic regions close to the telomeres and centromeres of plant chromosomes. By fluorescence *in situ* hybridization it was demonstrated that 158 bp repeat clusters of the *Sau*3A satellite I exclusively co-localize with many of the largest DAPI positive, paracentromeric, heterochromatic regions in *B. procumbens* (Fig. 4a–d). There is a remarkable chromosomal polymorphism regarding the number of sites. Two major and two faint centromeric clusters were detected reproducibly. The number of the remaining sites varied between six and eight, reflecting that this satellite family is probably not fully homogenized at the chromosomal level and the total number of arrays has not been fixed in *B. procumbens*. It has been suggested that different numbers of repeat clusters may represent intermediate or transition stages in the evolution of satellite DNA families [7, 58], although equally, random occurrence of the events leading to dispersion, amplification or deletion of the sequences may give the polymorphisms observed.

Tandem arrays of the *Sau*3A satellite II coincided with centromeres of sixteen chromosomes (Fig. 4a–d). Multi-colour hybridization enabled the assignment of the two major repeat clusters of *Sau*3A satellite families I and II to the same chromosome pair. Centromeric satellite repeats have been observed in many plants, and the actual size and hence length of the arrays varies often to a considerable extent between chromosomes of a complement [19, 31, 35]. Aside from their centromeric localization with substantial variation of copy number per hybridization site, the repeats of the *Sau*3A satellite family II were also found along all chromosome arms (Fig. 4e–f). The observed chromosomal interspersion is consistent with the pattern revealed by Southern hybridization (Fig. 2b). The mechanism of dispersion is not known, but might involve unequal exchange, transposition or gene conversion. The movement of a repetitive DNA sequence in *Oryza* species by transposition has recently been described [44].

Members of the *Sau*3A family III (pTS3) are interspersed and almost uniformly distributed over all *B. procumbens* chromosomes. pTS3-like repeats occur in clusters at a few chromosomal regions, and may be part of or homologous to a larger repeat amplified on these sites, consistent with the 1.5 kb *Bgl*II fragments detected in genomic Southern hybridization. Remarkable is the almost complete exclusion from the short arm of chromosome 3 (following the numbering of de Jong and Blom in [9]) which harbours the tandem arrays of the 18S-5.8S-25S rRNA genes. In *B. vulgaris*, absence from the nucleolus organizer region is a typical feature of *Ty*1-*copia*-like retrotransposons and of BNR1, a family of LINE-like non-LTR retrotransposons [53]. Both retrotransposon types have also been found in *Procumbentes* species (S. Kubis and T. Schmidt, unpublished results); however, no homology was detected between pTS3 and either of the retroelements. Dispersed repetitive DNA sequences which do not fully display the typical structures and features of mobile elements have been characterized in rye and barley [21, 45]. A homology search within the EMBL/GenBank data library did not reveal any homology to other transposable elements so that the spreading mechanism of pTS3 remains unclear.

In species of the genus *Beta*, the chromosomes are relatively small, similar in size and have an average DNA content of ca. 80–90 Mb per chromosome based on a haploid genome size of 750 Mb [4]. Fluorescence *in situ* hybridization to extended target sites of pachytene chromosomes enables the physical location of DNA sequences and determination of their order along the chromosomes with high resolution and has also been used for fine mapping of rRNA genes and tandemly repeated sequences of *Secale cereale* [1]. We performed a high-resolution physical mapping with two diverged repeats from the *Sau*3A

satellite I. Within the analysed monomers of this family, pTS6 shows the highest divergence, is the only one with an internal deletion, and the lowest sequence identity was found to pTS5. Divergence by base pair substitutions, deletions and insertions results in a gradient of homology (sequence identity) between variants of a satellite repeat population, and also affects and limits the definition of a sequence family (Fig. 1A). Sequence heterogeneity can be used to characterize individual variants or groups but is probably not a sufficient criterion to circumscribe a subfamily.

Under stringency conditions higher than the internal sequence homology the repeats pTS5 and pTS6 hybridized to six distinct sites on the haploid complement ($n = 9$), but one centromeric site showed a compound structure: the internal region, sharpely defined by red fluorescence and consisting of a long pTS5 array, is flanked on both sides by pTS6 clusters (green fluorescence) running into non-homologous genomic DNA sequences. Our results show clearly that pTS5 and pTS6 are not only variants but represent indeed different subfamilies that can be spatially separated. The localization of diverged satellite subfamilies in different intrachromosomal domains is novel and has not yet been reported in plants. The presence of three distinct alphoid DNA domains at the centromere of human chromosome 22 has been shown by *in situ* hybridization on extended chromatin [3]. Different subsets were also found on chromosome 17 of man [64], but subsets of alphoid satellite sequences in man are mostly specific for individual chromosomes [reviewed in 8]. In *D. melanogaster*, it has been shown that satellite DNAs, closely related in sequence, are often located near one another on the same chromosome [37].

The compound structure of a *Sau*3A satellite I repeat block indicates strongly that, in addition to the localization on separate chromosomes, the large scale organization of tandemly repeated DNA in plants includes co-existence of subsets in discrete intrachromosomal domains. It can be assumed that there are constraints where individual subfamilies are located, most likely caused by their amplification mechanisms like unequal cross-over of sister chromatids or slipped-strand replication (leading to sequence homogenization) since the domains were found on one chromosome [36]. The analysis of YAC and Lambda clones of alien *B. procumbens* chromatin in *B. vulgaris* has shown that repeats of the *Sau*3A satellite family I are interspersed with other wild beet sequences [32]. In contrast, in tomato it was proposed that large clusters of the TGRI satellite are uninterrupted [12, 34].

Nematode-resistant lines ($2n = 19$) of cultivated beet have been selected after interspecific crosses between *B. vulgaris* and *Procumbentes* species [summarized in 29]. Probes with homology to the *Sau*3A satellite family I have been intensively used for screening [52, 25] and fine mapping of *Procumbentes* chromatin towards the isolation of the nematode resistant gene [27, 32, 33, 46, 47]. The molecular investigations and the physical mapping of the three highly amplified repeat families described here might have implications for the selection of existing and future breeding material and its molecular analysis. Fluorescence *in situ* hybridization has shown that blocks of *Sau*3A satellite families II are co-located with blocks of the satellite family I in many centromeric regions and the interspersed sequence family pTS3 is dispersed throughout most of the *B. procumbens* genome. Hence, they might provide useful landmarks for the molecular analysis of introgressed wild beet chromatin. The Southern analysis within genomes of related species has shown that the three repeat families are specific for the section *Procumbentes*, and the cross-hybridization to *B. nana* is removable by stringent washing. Therefore, those sequences are, together with other section-specific satellites [50, 52], valuable markers for the evaluation of the genetic constitution in triple hybrids made by crosses of *Beta*, *Corollineae* and *Procumbentes* species.

De Jong *et al.* reported that the resistance gene-bearing *B. procumbens* chromosome in monosomic addition lines of cultivated beet has nucleolus organizer activity, and hence possesses the rRNA gene cluster [9, 10]. Our results show the major loci of the 18S-5.8S-25S rRNA genes are on the short arm of a chromosome pair, miss-

ing the *Sau*3A satellite I which is closely linked to a nematode resistance locus. A possible explanation is the existence of minor rDNA or satellite sites in *B. procumbens*; in sugar beet, minor rRNA gene clusters have been found in some mitotic metaphases [54], while polymorphisms in number of major loci of satellite are shown here (Fig. 4a–d).

The separation of megabase-sized DNA fragments of nematode-resistant cultivated beet lines by pulsed field gel electrophoresis, construction of YAC libraries and map-based cloning has enabled molecular analyses and physical mapping of long DNA stretches covering large regions of the introgressed wild beet chromosome segment [26, 32, 33, 46]. YAC clones containing blocks of repeats of *Sau*3A satellite family I have been isolated and characterized, and the size indicates that these long tandem arrays originate from genomic regions that can be detected and physically mapped by fluorescence *in situ* hybridization. Therefore, we propose that one of the genes conferring the resistance to the beet cyst nematode *H. schachtii* is located in close vicinity of the centromeres of a *B. procumbens* chromosome.

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References

1. Albini SM, Schwarzacher T: *In situ* localization of two repetitive DNA sequences to surface-spread pachytene chromosomes of rye. Genome 35: 551–559 (1992).
2. Anamthawat-Jonsson K, Heslop-Harrison JS: Isolation and characterization of genome-specific DNA sequences in Triticeae species. Mol Gen Genet 240: 151–158 (1993).
3. Antonacci R, Rocchi M, Archidiacono N, Bandini A: Ordered mapping of three alpha satellite DNA subsets on human chromosome 22. Chrom Res 3: 124–127 (1995).
4. Arumuganathan K, Earle ED: Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9: 208–218 (1991).
5. Bennetzen JL, Schrick K, Springer PS, Brown WE, San-Miguel P: Active maize genes are unmodified and flanked by diverse classes of modified, highly repetitive DNA. Genome 37: 565–576 (1994).
6. Brandes A, Jung C, Wricke G: Nematode resistance derived from wild beet and its meiotic stability in sugar beet. Plant Breed 99: 56-64 (1987).
7. Charlesworth B, Sniegowski P, Stephan W: The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 370: 215–220 (1994).
8. Choo KH, Vissel B, Nagy A, Earle E, Kalitsis P: A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and a derivation of a new consensus sequence. Nucl Acids Res 19: 1179–1182 (1991).
9. de Jong H: Investigation into chromosome morphology of sugar beet and related wild species. Ph.D. thesis, University of Amsterdam (1981).
10. de Jong JH, Speckmann GJ, de Bock TSM, Lange W, van Voorst A: Alien chromosome fragments conditioning resistance to beet cyst nematode in diploid descendants from monosomic additions of *Beta procumbens* to *B. vulgaris*. Can J Genet Cytol 28: 439–443 (1986).
11. Flavell RB, Bennet MD, Smith JB: Genome size and the proportion of repeated nucleotide sequence DNA in plants. Biochem Genet 12: 257–269 (1974).
12. Ganal MW, Broun P, Tanksley SD: Genetic mapping of tandemly repeated telomeric DNA sequences in tomato (*Lycopersicon esculentum*). Genomics 14: 444–448 (1992).
13. Ganal MW, Lapitan NLV, Tanksley SD: A molecular and cytogenetic survey of major repeated DNA sequences in tomato (*Lycopersicon esculentum*). Mol Gen Genet 213: 262–268 (1988).
14. Gerlach WL, Bedbrook JR: Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucl Acids Res 7: 1869–1885 (1979).
15. Grellet F, Delcasso F, Panabieres F, Delseny M: Organization and evolution of a higher plant alphoid-like satellite DNA sequence. J Mol Biol 187: 495–507 (1986).
16. Gruenbaum Y, Naveh-Many T, Cedar T, Razin A: Sequence specificity of methylation in higher plants. Nature 292: 860–862 (1981).
17. Gupta V, Jagannathan V, Lakshmikumaran MS: A novel AT-rich tandem repeat in *Brassica nigra*. Plant Sci 68: 233–229 (1990).
18. Hallden C, Bryngelsson T, Säll T, Gustafsson M: Distribution and evolution of a tandemly repeated DNA sequence in the family *Brassicaceae*. J Mol Evol 25: 318–323 (1987).
19. Harrison GE, Heslop-Harrison JS: Centromeric repetitive DNA sequences in the genus *Brassica*. Theor Appl Genet 90: 157–165 (1995).
20. Heijbroek W, Roelands AJ, de Jong JH, van Hulst C, Schoone AH, Munning RG: Sugar beets homozygous for resistance to beet cyst nematode (*Heterodera schachtii* Schm.) developed from monosomic additions of *Beta procumbens* to *B. vulgaris*. Euphytica 38: 121–131 (1988).
21. Hueros G, Loarce Y, Ferrer E: A structural and evolutionary analysis of a dispersed repetitive sequence. Plant Mol Biol 22: 635–643 (1993).

22. Hutchinson J, Londsdale DM: The chromosomal distribution of cloned highly repetitive sequences from hexaploid wheat. Heredity 48: 371–376 (1982).
23. Ingham LD, Hannah WW, Baier JW, Hannah LC: Origin of the main class of repetitive DNA in selected *Pennisetum* species. Mol Gen Genet 238: 350–356 (1993).
24. Iwabuchi M, Itoh K, Shimamoto K: Molecular and cytological characterization of repetitive DNA sequences in *Brassica*. Theor Appl Genet 81: 349–355 (1991).
25. Jung C, Herrmann RG: A DNA probe for rapid screening of sugar beet (*Beta vulgaris* L.) carrying extra chromosomes from wild beets of the *Procumbentes* section. Plant Breed 107: 275–279 (1991).
26. Jung C, Kleine M, Fischer F, Herrmann RG: Analysis of DNA from a *Beta procumbens* chromosome fragment in sugar beet carrying a gene for nematode resistance. Theor Appl Genet 79: 663–672 (1990).
27. Jung C, Koch R, Fischer F, Brandes A, Wricke G, Herrmann RG: DNA markers closely linked to nematode resistances genes in sugar beet (*Beta vulgaris* L.) mapped using chromosome additions and translocations originating from wild beets of the *Procumbentes* section. Mol Gen Genet 232: 271–278 (1992).
28. Jung C, Wricke G: Selection of diploid nematode-resistant sugar beet from monosomic addition lines. Plant Breed 98: 205–214 (1987).
29. Jung C: Breeding for nematode resistance in sugar beet. Ann Biol 3: 15–25 (1987).
30. Kamm A, Galasso I, Schmidt T, Heslop-Harrison JS: Analysis of a repetitive DNA family from *Arabidopsis arenosa* and relationship between *Arabidopsis* species. Plant Mol Biol 27: 853–862 (1995).
31. Kamm A, Schmidt T, Heslop-Harrison JS: Molecular and physical organization of highly repetitive, undermethylated DNA from *Pennisetum glaucum*. Mol Gen Genet 244: 420–425 (1994).
32. Kleine M, Cai D, Elbl C, Herrmann RG, Jung C: Physical mapping and cloning of a translocation in sugar beet (*Beta vulgaris* L.) carrying a gene for nematode (*Heterodera schachtii*) resistance from *B. procumbens*. Theor Appl Genet 90: 399–406 (1995).
33. Klein-Lankhorst RM, Salentijn EMJ, Dirkse WG, Arens-de Reuver M, Stiekema WJ: Construction of a YAC library from a *Beta vulgaris* fragment addition and isolation of a major satellite DNA cluster linked to the beet cyst nematode resistance locus $Hs1^{pat-1}$. Theor Appl Genet 89: 426–434 (1994).
34. Lapitan NLV, Ganal MW, Tanksley SD: Somatic karyotype of tomato based on *in situ* hybridization of the TGRI satellite repeat. Genome 32: 992–998 (1989).
35. Lapitan NLV: Organization and evolution of higher plant nuclear genomes. Genome 35: 171–181 (1991).
36. Levinson G, Gutman GA: Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol 4: 203–221 (1987).
37. Lohe AR, Hilliker AJ, Roberts PA: Mapping simple repeated DNA sequences in heterochromatin of *Drosophila melanogaster*. Genetics 134: 1149–1174 (1993).
38. Löptien H: Breeding nematode-resistant beets. I. Development of resistant alien additions by crosses between *Beta vulgaris* L. and wild species of the section *Patellaris*. Z Pflanzenzüchtg 92: 208–220 (1984).
39. Maluszynska J, Heslop-Harrison JS: Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J 1: 159–166 (1991).
40. McIntyre CL, Pereira S, Moran LB, Appels R: New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. Genome 33: 635–640 (1990).
41. Meinkoth J, Wahl G: Hybridization of nucleic acids immobilized on solid support. Anal Biochem 138: 267–284 (1984).
42. Nakamura C, Tsuchiya T: Nematode resistance and meiotic abnormality in diploid sugar beet selected from interspecific *Beta vulgaris* × *B. procumbens* hybrids. Plant Breed 100: 41–53 (1988).
43. Nelson M, Raschke E, McClelland M: Effect of site-specific methylation on restriction endonucleases and DNA modification methyltransferases. Nucl Acids Res 21: 3139–3154 (1993).
44. Ohtsubo H, Ohtsubo E: Involvement of transposition in dispersion of tandem repeat sequences (TrsA) in rice genomes. Mol Gen Genet 245: 449–455 (1994).
45. Rogowsky PM, Manning S, Liu SY, Landridge P: The R173 family of rye-specific DNA sequences: a structural analysis. Genome 34: 88–95 (1991).
46. Salentijn EMJ, Sandal NN, Klein-Lankhorst R, Lange W, De Bock TSM, Marcker KA, Stiekema WJ: Long-range organization of a satellite DNA family flanking the beet cyst nematode resistance locus (*Hs1*) on chromosome-1 of *B. patellaris* and *B. procumbens*. Theor Appl Genet 89: 459–466 (1994).
47. Salentijn EMJ, Sandal NN, Lange W, de Bock TSM, Krens FA, Marcker KA, Stiekema WJ: Isolation of DNA markers linked to a beet cyst nematode resistance locus in *Beta patellaris* and *Beta procumbens*. Mol Gen Genet 235: 432–440 (1992).
48. Sambrook J, Frisch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
49. Savitzky H: Hybridization between *Beta vulgaris* and *B. procumbens* and transmission of nematode (*Heterodera schachtii*) resistance to sugar beet. Can J Genet Cytol 17: 197–209 (1975).
50. Schmidt T, Heslop-Harrison JS: Variability and evolution of highly repeated DNA sequences of the genus *Beta*. Genome 36: 1074–1079 (1993).
51. Schmidt T, Jung C, Metzlaff M: Distribution and evolution of two satellite DNAs in the genus *Beta*. Theor Appl Gen 82: 793–799 (1991).
52. Schmidt T, Junghans H, Metzlaff M: Construction of *Beta procumbens*-specific DNA probes and their applica-

tion for the screening of *B. vulgaris* × *B. procumbens* ($2n = 19$) addition lines. Theor Appl Genet 79: 177–181 (1990).
53. Schmidt T, Kubis A, Heslop-Harrison JS: Analysis and chromosomal localization of retrotransposons in sugar beet (*Beta vulgaris* L.): LINEs and *Ty1-copia*-like elements as major components of the genome. Chrom Res 3: 335–345 (1995).
54. Schmidt T, Schwarzacher T, Heslop-Harrison JS: Physical mapping of rRNA genes by fluorescent *in-situ* hybridization and structural analysis of 5S rRNA genes and intergenic spacer sequences in sugar beet (*Beta vulgaris*). Theor Appl Genet 88: 629–636 (1994).
55. Schweizer G, Ganal M, Ninnemann H, Hemleben V: Species-specific DNA sequences for identification of somatic hybrids between *Lycopersicon esculentum* and *Solanum acaule*. Theor Appl Genet 75: 679–684 (1988).
56. Simeons CR, Gielen J, Van Montagu M, Inze D: Characterization of highly repetitive sequences of *Arabidopsis thaliana*. Nucl Acids Res 16: 6753–6766 (1988).
57. Singer MF: Highly repeated sequences in mammalian genomes. Int Rev Cytol 76: 67–112 (1982).
58. Strachan T, Webb D, Dover GA: Transition stages of molecular drive in multi-copy DNA families in *Drosophila*. EMBO J 4: 1701–1708 (1987).
59. Svitashev S, Bryngelsson T, Vershinin A, Pedersen C, Säll T, von Bothmer R: Phylogenetic analysis of the genus *Hordeum* using repetitive DNA sequences. Theor Appl Genet 89: 801–810 (1994).
60. van Geyt JPC, Oléo M, Lange W, de Bock TSM: Monosomic additions in beet (*Beta vulgaris*) carrying extra chromosomes of *Beta procumbens*. 1. Identification of the alien chromosomes with the help of isozyme markers. Theor Appl Genet 76: 577–586 (1988).
61. Vershinin AV, Svitashev SK, Gummesson PO, Salomon B, von bothmer R, Bryngelsson T: Characterization of a family of tandemly repeated DNA sequences in Triticeae. Theor Appl Genet 89: 217–225 (1994).
62. Vongs A, Kakutani T, Martienssen RA, Richards ER: *Arabidopsis thaliana* methylation mutants. Science 260: 1926–1928 (1993).
63. Wang ZX, Kurata N, Saji S, Katayose Y, Minobe Y: A chromosome 5-specific repetitive sequence in rice (*Oryza sativa* L.) Theor Appl Genet 90: 907–913 (1995).
64. Willard HF, Waye JS: Hierachic order in chromosome-specific alpha satellite DNA. Trends Genet 3: 192–198 (1987).
65. Wu T, Wu R: A novel repetitive DNA sequence in the genus *Oryza*. Theor Appl Genet 84: 136–144 (1992).
66. Zhang HB, Dvorák J: Isolation of repeated DNA sequences from *Lophophyrum elongatum* for detection of *Lophophyrum* chromatin in wheat genomes. Genome 33: 283–294 (1990).

Soybean Chromosome Painting: A Strategy for Somatic Cytogenetics

L. Shi, T. Zhu, M. Morgante, J. A. Rafalski, and P. Keim

Cytological identification of soybean mitotic metaphase chromosomes ($2n = 40$) has been severely limited by their small size and uniform karyomorphology. We have developed fluorescent in situ hybridization (FISH), PCR-primed in situ labeling (PCR-PRINS) procedures, and molecular probes for routine cytological identification and for the physical mapping of soybean somatic chromosomes. Chromosome preparation has been achieved by modifications of previous protocols and through the preparation of root-tip protoplasts prior to chromosome spreading. Initially our probe selection focused on highly repeated DNAs that provide very intense localized hybridization signals. Repetitive gene probes that have proven valuable include the rDNA loci (5S and 45S) which are chromosome specific. We have also developed satellite DNA probes for two different sequence families: the SB92 and the STR120 satellites. Both of these are tandemly arranged at multiple chromosomal loci. By using different cloned examples of each family, we have been able to selectively label unique subsets of soybean chromosomes. Double hybridization with biotin and digoxigenin labeled probes has allowed us to determine the chromosomal overlap between different probes. In addition, we have joined portions of the metaphase chromosome painting patterns with the genetic map by single-copy FISH and PCR-PRINS detection of the RFLP loci G8.15, G17.3, and A199a and A199b. Total genomic DNA in situ hybridization (GISH) patterns were also used to characterize the soybean chromosomes.

Historically, cytogenetic analysis has played an important role in establishing the physical basis of genetics and is increasingly important with the refinement of in situ hybridization technologies. For example, fluorescence in situ hybridization (FISH) allows the localization of particular DNA sequences on both condensed chromosomes and interphase nuclei (Heslop-Harrison 1991; Lichter and Ward 1990; Trask 1991). This technology can be used to map genes or anonymous DNA sequences onto chromosomes and therefore provide physical maps for comparison to genetic maps. This will refine our understanding of genome organization and genetic processes (e.g., Gustafson and Dille 1992). Signal intensity and detection had limited FISH to repeated DNA probes like ribosomal RNA genes, satellite DNA sequences, and larger unique DNA probes (Heslop-Harrison 1991; Lichter and Ward 1990; Trask 1991), but technical improvements are now allowing the use of smaller single-copy probes (Fan et al. 1990; Happell-Parton et al. 1994, Zhu et al. 1995b). Many genetic systems have a very large number of genetically mapped RFLP probes that can now be used for physical mapping (e.g., Happell-Parton et al. 1994). Primed in situ chromosome labeling (PRINS; Abbo et al. 1993; Gosden et al. 1991; Koch et al. 1989) has been accomplished by using unlabeled oligonucleotide primers in conjunction with labeled nucleotide triphosphates for in situ DNA synthesis. This approach is even more successful when coupled to thermocycling (i.e., PCR-PRINS), which increases signal more than 20% with each cycle (Terkelsen et al. 1993). PCR-PRINS can detect sequence tagged sites that are frequently less than 1 kbp in size. Broader patterns in genome structure and mapping are detectable through image analysis of genomic in situ hybridization (GISH) that provides a "visual Cot curve" of the genome. GISH analysis uses total genomic DNA as a FISH probe and easily identifies both foreign introgressed chromosomes and chromosomal structure associated with rapidly annealing DNAs (Anamthawat-Jónsson et al. 1990; Mukai et al. 1993).

Simultaneous cytogenetic mapping of

From the Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640 (Shi, Zhu, and Keim), the Dipartimento di Produzione Vegetale e Tecnologie Agrarie, Universita' di Udine, Via delle Scienze 208, I-33100 Udine, Italy (Morgante), and DuPont Ag Biotechnology, Wilmington, Delaware (Rafalski). L. Shi and T. Zhu are currently at the Section of Plant Biology, University of California, Davis, California. This work was supported by USDA grant no. 92-37300-7523, DOE-PER grant no. 9408009, NSF grant no. BSR-9107042, and the NAU Cowden Endowment.

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multiple loci provides direct comparisons of their physical locations on the same set of chromosomes. This has been accomplished through the image analysis of multicolor fluorescence in situ hybridization (MIS; Leitch et al. 1991; Ried et al. 1992). In its simplest form, MIS is accomplished when two different probes are labeled with either biotin or digoxigenin and then stained with fluorescent avidin or antibody. Improved image processing now allows spectral analysis of mixed fluorochrome signals such that greater than seven probes have been used simultaneously in MIS (Ried et al. 1992). In combination with traditional cytological "landmarks," MIS represents the definitive tool for the physical mapping of multiple genetic probes.

Recently, soybean genetic analysis has been accelerated through the use of molecular markers without the advantages of a coordinated cytogenetic map (Apuya et al. 1988; Keim et al. 1990; Shoemaker et al. 1992). This is due to the small size, the homogeneous morphology, and the relatively large number of soybean chromosomes (Palmer and Kilen 1987). Meiotic pachytene chromosome analysis developed by Singh and Hymowitz (1988) established a detailed soybean chromosome map, but it has not yet been joined with the genetic linkage groups. Joining cytogenetics with molecular genetics for chromosome analysis appears to be the best approach for developing a soybean cytogenetic map that is coordinated with the genetic linkage groups. In situ hybridization of DNA probes to soybean chromosomes was first reported by Skorupska et al. (1989) and later by Griffor et al. (1991). In both cases, the highly repeated 45S rRNA genes in the NOR region were targeted and single-copy genomic targets failed to be detected (Vodkin 1994).

In this article we present a comprehensive strategy for chromosome painting using different categories of DNA probes. In addition to repeating the 45S rDNA analysis using FISH, we present a new repertoire of molecular probes and improved methods adapted specifically for soybean somatic chromosome analysis. In addition to the two rDNA loci, we analyze two satellite DNAs families, single-copy RFLP probes, and sequence-tagged sites using PCR-PRINS. We describe a comprehensive strategy for uniquely "painting" soybean mitotic chromosomes for their identification and correlation to genetic maps.

Table 1. Soybean sequence probes

Probes	Category	Size (kbp)	Vector	Reference
TGM	Total Genomic DNA	—	—	Keim and Shoemaker 1988
pM4.9	5S rDNA	0.35	pUC9	Quemada et al. 1987
RB115	45S rDNA	8.0	Charon 4A	Jackson and Lark 1982
SB92	Repeated sequence	0.6	pBS+	Vahedian et al. 1995
STR120-A.1	Repeated sequence	0.365	pUC18	M. Morgante (unpublished data)
STR120-A.2	Repeated sequence	0.245	pBS SK−	M. Morgante (unpublished data)
STR120-A.3	Repeated sequence	0.365	pBS SK−	M. Morgante (unpublished data)
STR120-B.1	Repeated sequence	0.245	pUC18	M. Morgante (unpublished data)
G17.3	Large single copy	8.0	pUC9	Apuya et al. 1988
G8.15	Large single copy	4.0	pUC9	Apuya et al. 1988
A199	Small single copy	1.26	pBS+	Zhu et al. 1995a

Materials and Methods

Plant Materials and Chromosome Preparation

Soybean chromosomes were prepared from the cultivar BSR-101 ($2n = 2x = 40$) using isolated root-tip protoplasts according to Zhu et al. (1995b). Briefly, root tips synchronized by temperature shifts (Skorupska et al. 1989) are harvested and then fixed. The fixed tissue is treated with cellulytic enzymes to liberate individual protoplasts. Chromosomes are spread onto microscope slides by dropping a cellular suspension from about 30 cm, followed by flame drying.

DNA Probe Preparation

Eleven soybean DNA probes from five sequence complexity categories were selected for this study. The origins and properties of these probes are listed in Table 1. DNA probes were labeled by random hexamer extension with either biotin-16-dUTP or with digoxigenin-11-dUTP according to Zhu et al. (1995b).

In situ Hybridization

In situ hybridization was conducted according to Pinkel et al. (1986) with minor modifications (Zhu et al. 1995b). Briefly, chromosome spreads were denatured at 70°C in a 70% formamide and 2× SSC solution, and then dehydrated through a −20°C ethanol series. The probe hybridization solution consisted of 50% formamide, 2× SSC, 10% dextran sulphate, 50 μg/ml degraded herring sperm DNA and 2 μg/ml of denatured biotinylated (or dioxigeninylated) DNA probe. For double-probe experiments, 2 μg/ml of the biotinylated DNA probe and 2 μg/ml of the digoxigenin-11-dUTP labeled DNA probe were added to the hybridization solution. Hybridization was performed overnight at 37°C.

PCR-Primed in situ Labeling (PCR-PRINS)

Small single-copy sequences were labeled and detected by PCR-PRINS according to Terkelsen et al. (1993) and Gosden and Hanratty (1993) with modification (Zhu et al. 1995b). PCR-PRINS was conducted by adding 50 μl of labeling reaction mixture to the slide containing chromosome spreads at 95°C with a programmable thermal controller (MJ Research). The labeling reaction mixture contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1% Triton X-100, 1 mM $MgCl_2$, 0.2 mM dNTP mix with biotin-16-dUTP, 100 pmol of oligonucleotide primers (primer A199L-2: 5′-CGCACAACAAAACTGAGCTGCAAAGCCCG-3′ and primer A199R-2: 5′-ATGCTTCAAAGACATCAGAGACAATCAAGTCTGATGAAAG-3′; Zhu et al. 1995a). Fifteen units of *Taq* DNA polymerase were added after 5 min at 95°C. The reactions were thermocycled 30 times (1 min at 95°C, 2 min at 64°C, 10 min at 72°C). The reactions were stopped by adding 50 mM NaCl and 50 mM EDTA at pH 8.0 at 70°C.

Detection and Visualization of Hybridized Probes

After hybridization or PCR-PRINS, slides were rinsed twice in 50% formamide and 2× SSC, and thrice in PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% nonidet P-40, pH 8). The washes were performed at 45°C for high stringency and 37°C for low stringency treatments. If only a single biotinylated probe was used, slides were then incubated in PNM buffer (5% nonfat dry milk, 0.1% sodium azide in PN buffer) with 5 μg/ml fluorescein avidin DCS for 1 h at room temperature followed by multiple washes in PN buffer. When two differently labeled probes (biotin and digoxigenin) were used, the slides were incubated in PNM buffer with 5 pg/ml fluorescein avidin DCS and 20 μg/ml antidigoxigenin rhodamine for 1 h and washed in PN buffer. After rinsing slides were mounted in

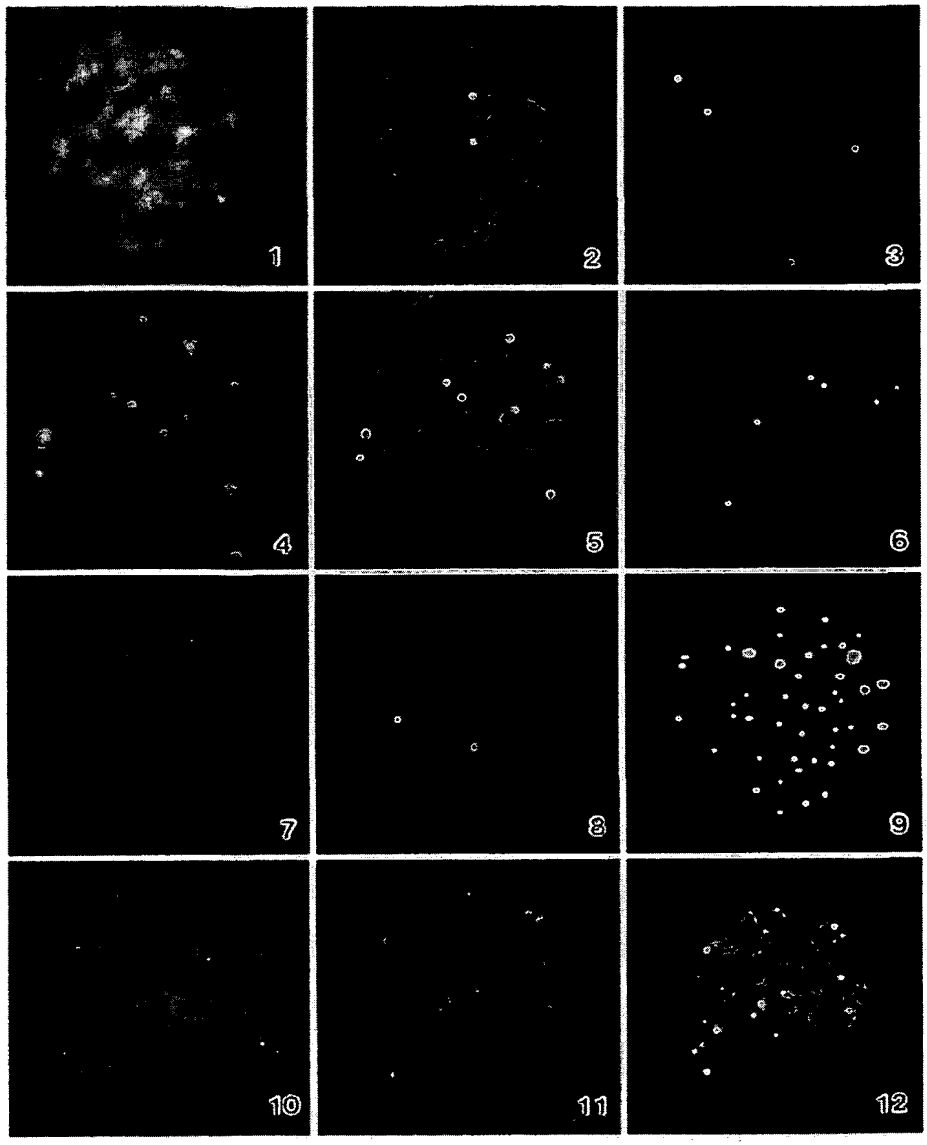

Figure 1. 5S rDNA FISH on metaphase chromosomes (FITC unprocessed image with propidium iodide counterstain).
Figure 2. 5S (red) and 45S (yellow) rDNA FISH on metaphase chromosomes (processed image). Note that only one locus (2 sites) was detected for each probe and that they are on separate chromosomes.
Figure 3. 5S rDNA (red) and the unique sequence G17.3 (yellow) FISH. Two sites for each probe are observed (processed image).
Figure 4. 5S rDNA and satellite DNA SB-92 FISH on metaphase chromosomes (rodamine and FITC unprocessed image without chromosomal counterstaining overlay).
Figure 5. 5S rDNA and satellite DNA SB-92 FISH on metaphase chromosomes (processed image with DAPI signal overlaid).
Figure 6. Satellite DNA STR120-B.1 FISH on metaphase chromosomes (processed image).
Figure 7. STR120-A.1 FISH (FITC unprocessed image). Two large diffuse low intensity hybridization sites are observable.
Figure 8. STR120-A.1 FISH (processed image with DAPI counterstain). This is the same nucleus as in Figure 7 with a mirror orientation.
Figure 9. STR120-A.2. FISH on metaphase chromosomes (processed image).
Figure 10. STR120-A.3 FISH on metaphase chromosomes (unprocessed image). FISH signal is from FITC labeling with propidium iodide counterstaining.
Figure 11. STR120-A.3 FISH on metaphase chromosomes (unprocessed image of the same nucleus in Figure 10). FITC signal with DAPI counterstaining reveals more minor sites than in Figure 10.
Figure 12. STR120-A.3 FISH on metaphase chromosomes (processed image). FISH signal from Figure 11 was superimposed onto DAPI counterstained chromosomes.

VECTASHIELD antifade medium (Vector Laboratories) containing 0.5 μg/ml propidium iodide or DAPI (4',6-diamidino-2-phenylindole).

Observations were made with a Zeiss Axioplan epifluorescence microscope with different combinations of Zeiss excitation and emission filters: 487709 for DAPI and 487701 for FITC and propidium iodide staining. Double-label signals were detected by the dual excitation and emission filter 51006. Images were recorded with Kodak Ektachrome ASA 400 color slide film.

Image Processing

Two color slides with the chromosome image and with the hybridization signals from the same chromosome spread were scanned into an Apple Macintosh computer-based image processing system using a Epson ES-600C scanner. The two images were aligned, the contrast adjusted and then they were recolored with the Adobe Photoshop software. Processed images were recorded on Fujichrome ASA 100 color slide film with a Polaroid CI-5000 digital palette film recorder.

Results

The goal of this work has been to develop diverse DNA sequences for the mitotic identification of soybean chromosomes. It is necessary to use different experimental approaches for single-copy versus highly repetitive sequences due to the large differences in signal intensity generated for each. In this article, we illustrate the utility of in situ chromosome analysis for soybean by presenting examples of different DNA probes representative of particular sequence categories. We also show how they can be used in combinations (MIS) for chromosomal comparison and for coordinating genetic linkage maps with cytogenetic maps.

rDNA Sequences

As high-copy tandemly repeated genes, rDNA sequences provide strong localized hybridization signals. Soybean has only one 5S rDNA locus (i.e., two hybridizing sites) and one 45S rDNA locus as observed in both interphase and metaphase nuclei (Skorupska et al. 1989; Figures 1–5). We observed hybridization signal in about 70% of interphase nuclei and 50% of metaphase mitotic nuclei. With low stringent washes, two additional minor hybridization sites can be detected by the 5S rDNA probe (data not shown). In metaphase chromosome spreads, both 5S and 45S

rDNA loci are observed at the distal regions of different chromosomes (Figures 1–5). When 5S and 45S rDNA loci were examined simultaneously with different fluorescent labels, it was clear that these loci are located on different chromosomes (Figure 2). Thus, the rDNA loci provide convenient high-intensity markers for two soybean chromosomes.

Satellite DNA Sequences

The SB92 satellite sequences are a 92 bp tandemly arranged element with about 10^5 copies (Kolchinsky and Gresshoff 1995; Vahedian et al. 1995). The SB92 probe pNAU401 revealed two major (large and more intense) and two or three minor (small and less intense) genomic locations in soybean chromosomes (Figures 5, 14, 20).

The STR120 satellite sequences are tandemly arranged about 120 bp repeated DNA elements (Morgante M, unpublished data). Four divergent STR120 probes were used for this study: STR120-A.1, STR120-A.2, STR120-A.3, and STR120-B.1. These cloned sequences ranged in homology from 60% to 90% (Morgante M, unpublished data). Two of the probes (STR120-A.1 and STR120-B.1) detected relatively simple hybridization patterns. STR120-A.1 consistently detected two large but low-intensity sites in the distal region of a single chromosome pair (Figures 7, 8, 13), while STR120-B.1 revealed four to eight sites located on distal ends of chromosomal arms (Figure 6). In contrast, probes STR120-A.2 and STR120-A.3 detected relatively complex hybridization patterns with sites on many chromosomes. The major hybridization sites were very repeatable, while the number of minor sites varied depending on the hybridization stringency used. Probe STR120-A.3 detected 14 major sites and about 10 minor sites (Figures 10–12). The major sites were distributed over six pairs of chromosomes (Figures 10–12). Probe STR120-A.2 detected an extremely complex pattern of more than 50 sites with major and minor sites distributed on all but one or two chromosomes (Figure 9).

Single-Copy DNA Sequences

Two relatively large anonymous single-copy DNA sequences were successfully detected by FISH. These probes, G8.15 and G17.3 (4 and 8 kbp, respectively), readily marked their homologous regions on the chromosomes using the standard FISH methodology. G8.15 and G17.3 each detected two sites on a single chromosome pair (Figure 15 and Figures 3 and 16, respectively). Both of these sequences had been genetically mapped in previous studies (Keim et al. 1990, Shoemaker et al. 1992) to linkage groups J (G8.15) and L (G17.3).

PCR-PRINS can detect relatively small chromosomal regions that cannot be observed using standard FISH protocols. Repeated efforts using the standard FISH (Pinkel et al. 1986), one-step PRINS (Koch et al. 1989, 1992), and PCR-IS (Gosden and Hanratty 1993) protocols failed to detect the duplicate *A-199* loci. The probe A199 is only 1260 bp which is evidently insufficient for FISH and simple PRINS detection in our study. In contrast, PCR-PRINS succeeded at frequently detecting four sites on two pairs of chromosomes (Figure 17). Parallel PCR-IS control experiments where no primers were added to the in situ cocktail resulted in no chromosomal signal (data not shown). Two of the detected sites were located in a distal chromosomal region, while the others were more internal. Although the *A199a* and *A199b* loci have only 90% sequence similarity (Zhu T, Shi L, and Keim P, unpublished data), the result of four sites is expected because the primers themselves are 100% homologous to both loci. The *A199a* and *A199b* loci have been genetically mapped in linkage groups K and J, respectively (Keim et al. 1990; Shoemaker et al. 1992) and the chromosomal locations are consistent with the genetic map positions.

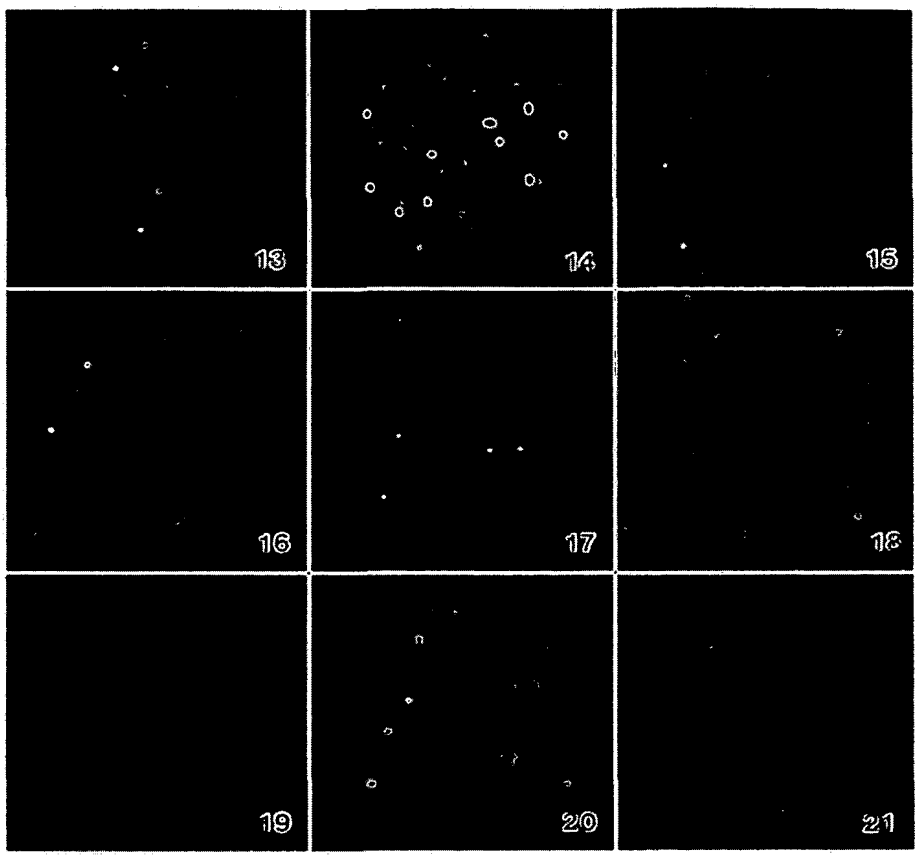

Figure 13. STR120-A.1 (yellow) and STR120-B.1 (red) MIS FISH on metaphase chromosomes (processed image).
Figure 14. STR120-A.3 and SB-92 MIS FISH on metaphase chromosomes (processed image). The 10 SB92 hybridization sites were at the same positions as 10 of the ST120-A.3 sites (yellow). Additional unique STR120-A.3 sites were observed and are shown in red.
Figure 15. G17.3 FISH on metaphase chromosomes (processed image with propidium iodide counterstain).
Figure 16. G8.15 FISH on metaphase chromosomes (processed image with propidium iodide counterstain).
Figure 17. PCR-PRINS at A199a and b on metaphase chromosomes (processed image).
Figure 18. GISH on metaphase chromosomes (FITC signal, unprocessed image).
Figure 19. DAPI counterstaining of metaphase chromosome (unprocessed image). Note that this is the same nucleus shown in Figure 1.
Figure 20. Total genomic DNA (red) and SB-92 (yellow) probes in MIS FISH (unprocessed images).
Figure 21. DAPI counterstain signal of metaphase chromosome. Note that this is the same nucleus shown in Figure 20.

GISH Patterns

Total genomic DNA was used as a probe against metaphase chromosome spreads in order to detect highly repetitive regions. Almost every soybean chromosome was labeled by the relatively large but less intensive GISH signal (Figures 18–21). However, the hybridization pattern was different among the chromosomes in terms of the signal area, intensity, and location. Ten to 13 chromosome pairs had relatively strong and large hybridization signals, while five to seven pairs of chromosomes were labeled only weakly (Figures 18 and 19). Large chromatin blocks within the chromosome arms were the most common and intense hybridizing locations, while telomeres did not hybridize as intensely as other chromosomal locations (Figures 18 and 19). Future image analysis of GISH will provide diagnostic patterns for many soybean chromosomes.

Double-Probe Hybridization

Several double-probe hybridization experiments were conducted to confirm the reliability of the techniques, determine the relative genomic arrangements of probes, and test our capacity to use different types of probes in the same protocols. We used a variety of probe combinations including high-copy gene + repetitive DNA probes, high-copy gene + large single-copy DNA probes, repetitive DNA + repetitive DNA probes, high-copy gene + high-copy gene probes, and GISH + repetitive DNA probes. All of the hybridizations successfully detected signal from both probes (Figures 2–5, 13, 14, 20, and 21). There appears to be no technical limitations on mixing diverse probes in a single FISH protocol. Most sequences homologous to the probes used in the double-probe experiments had unique chromosomal locations. However, two probes (STR120A.2 and STR120A.3) from the satellite DNA family STR120 detected common locations, as evidenced by their partial overlap in hybridization sites (data not shown). In addition, the satellite sequences SB92 and STR120A.3 also have some proximal chromosomal locations (data not presented).

Counter-Staining Chromosome Images in FISH and PCR-PRINS

Both propidium iodide and DAPI are frequently used as counter-stains for chromosomal images in FISH and PCR-PRINS. However, in our studies propidium iodide staining was found to mask some low-intensity FISH sites. For instance, only eight major sites of the repetitive sequence STR120-A3 were detected with propidium iodide counter-staining (Figure 10), while more than 20 sites were observed with the DAPI counter-staining under the same hybridization condition (Figures 11 and 12). The A199 PCR-PRINS experiments generated reproducible but weak hybridization signals which were masked with propidium iodide but not DAPI (data not shown). This result agrees with the findings in FISH studies of other plant species (Heslop-Harrison et al. 1991). Hence, DAPI counter-staining seems necessary for detecting weak hybridization signal generated from small single copy or from minor repetitive DNA sites.

Discussion

The classical genetic model systems such as *Drosophila* and maize had many experimental advantages including highly resolved cytogenetic maps. Sophisticated molecular genetic analysis is now common to many diverse and genetically difficult organisms, but detailed cytogenetic maps are frequently not available. FISH and related technologies can now complement the genetic development of many systems by providing detailed cytogenetic information.

Soybean is an example of an intensely studied organism that can benefit from enhanced cytogenetic analysis using molecular and cytological technology. Identification and manipulation of somatic soybean chromosomes is essentially impossible based on their physical characters. Most of the soybean DNA sequences used in this study can be detected by simple FISH procedures. This includes total genomic DNA, rDNAs, satellite DNA sequences, and relatively large single-copy sequences. The smaller single-copy sequences (about 1 kbp) from RFLP loci such as *A199* could not be detected by this method. However, their detection was possible using a modified PCRPRINS protocol. Probes can be used in combinations and stained differentially to provide direct comparisons of their genomic locations. Because we have demonstrated the utility of many types of genomic sequences, it will now be possible for soybean researchers to develop somatic cytogenetic maps and to unite them with the genetic linkage map and its wealth of information. Routine somatic chromosome identification tools are the essential first step towards the eventual physical isolation of soybean chromosomes using flow cytometry or microscopic manipulation (Weber and Greulich 1992).

Repetitive DNA Sequences as Chromosomal Markers

Repetitive DNA sequences comprise high proportions of plant genomes and may account for nearly 60% of soybean DNA (Goldberg 1978; Gurley et al. 1979). Many of these repeated sequences are satellite DNA sequences that are so rapidly evolving that they are unique to the annual soybeans and even to specific chromosomes (Vahedian et al. 1995; Morgante M, unpublished data). Therefore, repetitive DNAs can be of great importance in genomic characterization and the investigation of genomic relationships. The strong hybridization signals generated by these probes provide the strong tags necessary for chromosome isolation (e.g., Lazer-Tweezers; Weber and Greulich 1992). Repetitive DNAs can also be detected by GISH, a special application of FISH where total genomic DNA is used as a probe. GISH detects all repetitive DNA families in the entire genome with a single probe providing a "visual C_0t curve" of a chromosome. The distribution of these high-copy sequences provides a distinct signal pattern on about half of the soybean chromosomes (Figures 17 and 18).

Single-Copy DNA Sequences as Chromosomal Markers

While repetitive DNAs provide robust cytological landmarks, they are difficult to map genetically. Unification of the cytological and genetic linkage maps will be primarily accomplished using single-copy DNA probes. These probes are widely used as molecular genetic markers and the soybean genetic map contains several hundred examples (Keim et al. 1990, Shoemaker et al. 1992). A few of these probes are relatively large (i.e., >4 kbp; Apuya et al. 1988) and can be detected with FISH alone (Figure 3). However, most are small (i.e., 1–2 kbp; Keim and Shoemaker 1988) and appear to require PCR-PRINS for detection. In our studies, the *A199* duplicate loci (about 1.2 kbp) were detected only with double-primer repeated PRINS-PCR (Terkelsen et al. 1993), while other approaches such as FISH (Pinkel et al. 1986), one-step PRINS with double primers (Koch et al. 1989, 1992), and PCR-IS with a single primer (Gosden and Hanratty 1993) failed. The capacity to detect small sequence tagged sites will allow the cytogenetic mapping of the large number of RFLP markers that have been developed for soybean. Again, this will bridge the cytogenetics and molecular genetics in soybean.

MIS of Chromosomal Markers

While single-label FISH has been used successfully to map individual probes to chromosome bands in both human and plant systems (Fan et al. 1990; Heslop-Harrison 1991; Trask 1991), this technique does not determine the relative order of different probes to the same chromosomes or even if the same chromosome is involved. Recently developed MIS can overcome these limitation by using probes with repeat sequences (Hindkjær et al. 1994; Leitch et al. 1991, Ried et al. 1992) and even small single-copy probes (Happell-Parton et al. 1994) in chromosome mapping. In soybean where individual somatic chromosomes are nearly indistinguishable, MIS becomes essential for assigning markers to the same chromosome. Furthermore, physical ordering of loci within a chromosome will be possible as MIS is expanded to greater than two probes (Ried et al. 1992).

Chromosomal Markers in Physical Mapping

Physical genomic mapping has succeeded by using very large DNA cloning methods (e.g., YACs or BACs), pulsed-field electrophoresis, and in situ analysis of metaphase chromosomes. Higher resolution in situ techniques have recently been applied to interphase or prophase nuclei (Bendriff et al. 1991; Trask 1991; Trask et al. 1989) and free chromatin (Albini and Schwarzacher 1992; Heng et al. 1992). These approaches allow physical maps with high resolution to be constructed (10–100 kbp; Inazawa et al. 1994). It is now possible to use FISH methods to map probes to chromosomes, subchromosomal regions, and even within 50 kbp region.

In the present study, we used 11 probes from different types of DNA sequences to tag and characterize soybean chromosomes. All 40 soybean chromosomes were tagged by FISH, GISH, or PCR-PRINS in either positive or the negative labeling. Among these, 36 chromosomes were labeled by repetitive DNA probes, while eight chromosomes were tagged by single-copy sequences. In addition, more than 10 chromosomes were negatively labeled by repetitive sequences or total genomic DNA. Additional studies are needed to provide a larger number of chromosome specific probes, but this study provides a strategy and methodology for this goal.

References

Abbo S, Miller TE, and King IP, 1993. Primer-induced in situ hybridization of plant chromosomes. Genome 36: 815–817.

Albini SM and Schwaracher T, 1992. In situ localization of two repetitive DNA sequences to surface-spread pachytene chromosomes of rye. Genome 35:551–559.

Anamthawat-Jónsson K, Schwarzacher T, Leitch AR, Bennett MD, and Heslop-Harrison JS, 1990. Discrimination between closely related Triticeae species using genomic DNA as a probe. Theor Appl Genet 79:721–728.

Apuya NR, Frazier BL, Keim P, Roth EJ, and Lark KG, 1988. Restriction fragment length polymorphisms as genetic markers in soybean, *Glycine max* (L.) Merrill. Theor Appl Genet 75:889–901.

Bendriff B, Gordon L, and Trask B, 1991. A new system for high-resolution DNA sequence mapping in interphase pronuclei. Genomics 10:75–82.

Fan Y-S, Davis L, and Show T, 1990. Mapping small DNA sequences by fluorescence in situ hybridization directly on banded metaphase chromosomes. Proc Natl Acad Sci USA 87:6223–6227.

Goldberg RB, 1978. DNA sequence organization in the soybean plant. Biochem Genet 16:45–68.

Gosden J and Hanratty D, 1993. PCR in situ: a rapid alternative to in situ hybridization for mapping short, low copy number sequences without isotopes. BioTechniques 15:78–80.

Gosden J, Hanratty D, Starling J, Fantes J, Mitchell A, and Porteous D, 1991. Oligonucleotide primed in situ DNA synthesis (PRINS): a method for chromosome mapping, banding, and investigation of sequence organization. Cytogenet Cell Genet 57:100–104.

Griffor MC, Vodkin LO, Singh RJ, and Hymowitz T, 1991. Fluorescent in situ hybridization to soybean metaphase chromosomes. Plant Mol Biol 17:101–109.

Gurley WB, Hepburn AG, and Key JL, 1979. Sequence organization of the soybean genome. Biochem Biophys Acta 561:167–183.

Gustafson JP and Dille JE, 1992. The chromosomal location of *Oryza sativa* recombination linkage groups. Proc Natl Acad Sci USA 89:8646–8650.

Happell-Parton AC, Albertson DG, Fishpool R, and Rabbitts PH, 1994. Multicolour fluorescence in situ hybridization to order small, single-copy probes on metaphase chromosomes. Cytogenet Cell Genet 66:42–47.

Heng HH, Squire J, and Tsui LC, 1992. High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc Natl Acad Sci USA 89:9509–9513.

Heslop-Harrison JS, 1991. The molecular cytogenetics of plants. J Cell Sci 100:15–21.

Heslop-Harrison JS, Schwarzacher T, Anamthawat-Jónsson K, Leitch AR, Shi M, and Leitch IJ, 1991. In situ hybridization with automated chromosome denaturation. Technique 3:104–115.

Hindkjær J, Koch J, Terkelsen C, Brandt CA, Kølvraa S, and Boland L, 1994, Fast, sensitive multicolor detection of nucleic acids in situ by primed in situ labeling (PRINS). Cytogenet Cell Genet 66:152–154.

Inazawa J, Ariyama T, Toidno T, Tanigami A, Nakamura Y, and Abe T, 1994. High resolution ordering of DNA markers by multi-color fluorescent in situ hybridization of prophase chromosomes. Cytogenet Cell Genet 65:130-135.

Jackson PJ and Lark KG, 1982. Ribosomal RNA synthesis in soybean suspension cultures growing in different media. Plant Physiol 69:234–239.

Keim P, Diers B, Olson T, and Shoemaker RC, 1990. RFLP mapping in soybean: association between marker loci and variation in quantitative traits. Genetics 126:735–742.

Keim P and Shoemaker RC, 1988. Construction of a random recombinant DNA library that is primarily single-copy sequence. Soybean Genet Newslett 15:147–148.

Koch JE, Kølvraa S, Peteersen KB, Gregersen N, and Bolund L, 1989. Oligonucleotide priming methods for the chromosome-specific labeling of alpha satellite DNA in situ. Chromosoma 98:259–265.

Koch J, Mogensen S, Paderen S, Fischer H, Hindkjær J, Kølvraa S, and Bolund L, 1992. Fast one-step procedure for the detection of nucleic acids in situ by primer-induced sequence-specific labeling with fluorescein-12-dUTP. Cytogenet Cell Genet 60:1–3.

Kokchinsky A and Gresshoff PM, 1995. A major satellite DNA of soybean: 92 base pair tandem repeat. Theor Appl Genet 90:621–626.

Leitch IJ, Leitch AR, and Heslop-Harrison JS, 1991. Physical mapping of plant DNA sequences by simultaneous in situ hybridization of two differently labeled probes. Genome 34:329–333.

Lichter W and Ward DC, 1990. Is non-isotopic in situ hybridization finally coming of age? Nature 345:93–95.

Mukai Y, Nakahara Y, and Yamamoto M, 1993. Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome 36:489–494.

Palmer RG and Kilen TC, 1987. Quantitative genetics and cytogenetics. In: Soybeans: Improvement, production, and uses, 2nd ed (Wilcox JR, ed). Agron Ser. 16. Madison, Wisconsin: ASA, CSSA, SSSA; 135–209.

Pinkel D, Straume T, and Gray JW, 1986. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938.

Quemada H, Roth EJ, and Lark KG, 1987. Changes in methylation of tissue cultured soybean cells detected by digestion with the restriction enzymes *Hpa*II and *Msp*I. Plant Cell Rep 6:63–66.

Ried T, Baldini A, Rand TC, and Ward DC, 1992. Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy. Proc Natl Acad Sci USA 89:1388–1392.

Shoemaker RC, Guffy RD, Lorenzen LL, and Specht JE, 1992. Molecular genetic mapping of soybean: map utilization. Crop Sci 32:1091–1098.

Singh RJ and Hymowitz T, 1988. The genomic relationship between *Glycine max* (L.) Merr. and *G. Soja* Sieb. and Zucc. as revealed by pachytene chromosome analysis. Theor Appl Genet 76:705–711.

Skorupska H, Albertsen MC, Langholz KD, and Palmer RG, 1989. Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by in situ hybridization. Genome 32:1091–1095.

Terkelsen C, Koch J, Kølvraa S, Hindkjær J, Pederson S, and Bolund L, 1993. Repeated primed in situ labeling: formation and labeling of specific DNA sequences in chromosomes and nuclei. Cytogenet Cell Genet 63:235–237.

Trask BJ, 1991. Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. Trends Genet 7:149–154.

Trask B, Pinkle D, and Van den Engh G, 1989. The proximity of DNA sequence in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. Genomics 5:710–719

Vahedian M, Shi L, Zhu T, Oldmoto R, Danna K, and Keim P, 1995. Genomic organization and evolution of the soybean SB92 satellite sequence. Plant Mol Biol 29:857–862.

Vodkin LO, 1994. Molecular exploitation of soybean genetic resources. In: Plant genome analysis (Gresshoff PM, ed). Ann Arbor, Michigan: CRC Press; 83–96.

Weber G, Greulich KO, 1992. Manipulation of cells, organelles, and genomes by laser microbeam and optical trap. Int Rev Cytol 133:1–41.

Zhu T, Shi L, Doyle JJ, and Keim P, 1995a. Phylogenetic analysis of DNA sequence of a single nuclear locus in soybean and relatives. Theor Appl Genet 90:991–999.

Zhu T, Shi L, and Keim P, 1995b. Detection by in-situ fluorescence of short, single-copy sequences of chromosomal DNA. Plant Mol Biol Rep 13:270–277.

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λCM8, a human sequence with putative centromeric function, does not map to the centromere but is present in one to two copies at 9qter

Niolette I.McGill, Judith Fantes and Howard Cooke*

Medical Research Council Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

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ABSTRACT

A DNA fragment isolated from a human genomic library, was reported to be present at all human centromeres and present at 16–32 copies per genome. Reintroduction of this DNA into mammalian cells as a concatenated phage clone gave rise to dicentric chromosomes which gave rise to a new, stable, chromosome. Taken together these observations could mean that this DNA is part of a native centromere. We have re-examined the location and copy number of this sequence and find it to be present at 1–2 copies per genome with a single site of *in situ* hybridisation at 9qter.

INTRODUCTION

The mammalian kinetochore is a complex assembly of DNA and protein. A few of the protein components have been characterised using either naturally occurring polyclonal antisera from patients with a variety of auto immune diseases or monoclonal antibodies (1). At the level of the DNA there is even less information; mammalian centromeres are generally associated with tandemly repeated DNA sequences and two of these, the human alphoid sequence and the *Mus musculis* minor satellite, contain a DNA sequence which is recognised by the best characterised of the centromeric proteins CENPB (see ref 2 for a review). CENPB can be found at inactive centromeres (3) whereas CENP C (4) is found at active centromeres (3) and is believed to be a DNA binding protein (W.Earnshaw, pers com).

Complete sequence data and genetic test systems are not currently available for mammalian centromeres. It therefore remains possible that mammalian centromere function could be dependent on a DNA sequence different to and perhaps smaller than, the megabases of satellite DNAs found at centromeres. This is an attractive prospect since the possibility of creating mammalian artificial chromosomes would be made much simpler with a centromere of manageable size.

The isolation of a DNA fragment, apparently originating from human centromeres and with apparent centromere function, by Hadlaczky et al (5) has stimulated much interest. We have attempted to confirm the original report of the location and abundance of this sequence but we find a terminal location of a single copy of the sequence on chromosome 9.

RESULTS

We initially used the entire λCM8 for FISH analysis on normal human lymphocytes. A signal was seen on chromosome 9q ter. (Fig 1a.) Since this was in contrast to the localisation reported, we were concerned that there could be a difference due either to our use of the normal karyotype rather than that of the Colo 320 cells, used by Hadlaczky *et al* (5) or due to the competitor DNA used to suppress signal from the Alu repeat. This competitor DNA could perhaps block centromeric hybridisation of other parts of the sequence which may be repetitive. To facilitate DNA production and analysis, we subcloned λCM8 into pUC19 as Eco RI fragments. A probe combination excluding the Eco RI fragment containing the Alu repeat was also used for FISH analysis without suppression. The same signal was observed on 9q ter. (Fig 1b) FISH using the same probe combination or λCM8 on HeLa metaphase spreads showed a localisation identical to that in the normal lymphocytes (Fig 1c). The result obtained was again consistent with a localisation on chromosome 9q ter. When λCM8 was used without competition a typical Alu banding pattern was observed with little or no hybridisation at centromeres.(Fig 1d)

Eco RI inserts of pCM8 38, 25 & 26 and the Eco RI-Xho I fragment of pCM8-30 (which excluded the Alu repeat) were used to probe a Southern blot containing 6 EcoRI digested human genomic DNA's, the hybrid cell line 64063, a chinese hamster ovary/human hybrid line containing only 9q as a human component and known amounts of the plasmid subclones (fig 2). For all of the subclones the measured copy number was close to one per haploid genome and the appropriate signal was detected in the 9q containing hybrid but not in CHO DNA.

DISCUSSION

Requirements for a human centromeric DNA sequence include its presence at a human centromere. In the yeasts *S. cerevisiae* and *S.pombe* where the centromeric DNA can be isolated and tested for function there are related sequences at all centromeres (6). It would be reasonable to expect sequence relationships between mammalian centromeres, at least within a species. Alphoid DNA satisfies these expectations and for these reasons in addition to the binding of CENP B, remains a likely candidate

* To whom correspondence should be addressed

Figure 1. In situ hybridisation of λCM8 probe to metaphases from peripheral blood lymphocytes (a, b&d) and Hela cells (c). In figures 1a&c, signals from repeat sequences were suppressed using cot-1 DNA . A hybridisation signal is seen at 9q ter in both homologues in each cell. In figure (d) no suppression was used. Figure (b) shows in situ hybridisation of λCM8 probe without the Alu sequence without suppression.

centromere sequence. Although alphoid DNA mimics some aspects of centromere behaviour when reintroduced into a mammalian cell (7) it must remain a candidate since definitive functional evidence is lacking.

In contrast our data exclude λCM8 from any human centromere and are entirely consistent with a single copy of the sequence being present at 9qter. This contradicts the earlier findings (5) of Hadlaczky et al but they were unable to localise the sequence by FISH, relying on ^{3}H in situ hybridisation which was not convincing. The original paper did not present data to support the copy number of 16 to 32 per genome.

This sequence can not be a component of a normal human centromere and it can not have dominant centromere activity at its native location. We have not repeated the transformation experiments of Hadlaczky et al. The simplest explanation of the apparent ability of λCM8 to function as a centromere is that any biological activity that this sequence may demonstrate is a function of either its environment as a transgenome or its tandem organisation in the transformed cell lines.

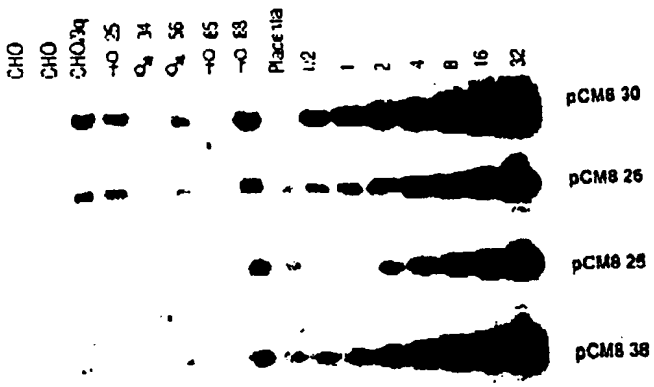

Figure 2. Hybridisation of Southern blot with the internal Eco RI fragments of λCM8. From the left lanes contain Eco RI digests of the following DNA's: 1 and 2 chinese hamster ovary, 3 9q containing hybrid, 4–9 human DNA's and lanes 10 to 16 dilutions of subclones of λCM8. These dilutions correspond to 1/2–32 copies of the fragments per diploid genome relative to the amounts of total human DNA loaded in tracks 4–9. The fragment sizes of the clones used as probes are, panel a) 4366 bp, b) 1859 bp, c) 808 bp and d) 440 bp.

MATERIALS AND METHODS

Subcloning CM8 insert

λCM8 DNA was digested with EcoRI and subcloned into EcoRI digested and dephosphorylated pUC19 (8). Subclones which corresponded in size and restriction map to the internal EcoRI fragments of λCM8 were selected for copy number analysis. We have called these pCM8 38, pCM8 25, pCM8 26 and pCM8 30. These subclones correspond to pCM1 (440bp) pCM6 (808bp) pCM11 (1859bp) and pk1620 (4366bp) respectively (see ref 5 fig 2).All enzymes were used according to manufacturers recommendations. DNA was electroporated into *E.coli* XL-1-Blue cells.

Isolation of DNA

Genomic DNA was prepared from human tissue samples which had been homogenised in S.E. buffer (75mM NaCl, 25mM EDTA pH 8).Proteinase k was added to 100μg/ml and SDS to 2% .The cell suspension was incubated overnight at 50°C followed by 3 phenol/chloroform/isoamyl alcohol(24:23:1) extractions and the DNA precipitated. Plasmid DNA's were made by standard CsCl/Ethidium gradient methods. DNA from the hybrid 64063 (which contained only chromosome arm 9q as a human component on a CHO background (9)) was isolated using a modified version of the method described in reference 10.

Copy number analysis

The copy number equivalents of pCM8 38,25,26 &30 were calculated on the basis of their measured sizes and that of the the human haploid genome (3×10^9 bp). The calculated single copy equivalents ranged from 10–22pg's depending on the size of the clone. The concentration of DNA in Eco RI digests of the plasmids was determined using fluorimetry with Hoechst 33258 as a DNA specific fluorochrome. Serial dilutions giving a range of 1/2–32 copy number equivalents were used to give the appropriate amount of DNA for loading on the gel.

Electrophoresis/Southern blotting

10 μg of each of the 6 genomic DNA samples used for copy number analysis were run on a 0.8% agarose gel run at 30v overnight. Prior to blotting, the DNA was UV nicked in the gel. DNA was then transferred onto Hybond N+(Amersham) and fixed by washing in 0.4M NaOH for 15 minutes followed by neutralisation.

Purification of gel fragments

Subclones were run on a 0.8% Gibco ultra pure low melting point agarose .DNA from inserts pCM8 25, 26and 30 was extracted using the Bio101 Gene clean kit.The smallest fragment from subclone pCM8 38 was extracted using the Bio101 Mermaid kit for maximum yield.

Hybridisation

The filter was hybridised as in (11) with 0.5% dried milk powder as a blocking agent. 100ng of each probe was labelled with 50μCi (α ^{32}P) d C.T.P. using the random primer method. Filters were washed in 2×SSC. at room temp, for 15' followed by three 15' washes in 0.1×SSC/ 0.1 S.D.S 65°C.

***In situ* hybridisation**

Biotin labelled nick translated probes were hybridised to metaphase chromosomes from peripheral blood lymphocytes or HeLa cells using standard methods (12). Where supression of repeated sequences was used probes were preannealed at 37°C in 50% formamide, 2×SSC 10% dextran sulphate with human Cot 1 DNA at 0.5 mg/ml for 15'. In hybridisations with the mixture of CM8 subclones without the Alu sequence, t-RNA was used as carrier and blocking DNA rather than salmon sperm DNA. The slides were counterstained with propidium iodide (1μg/ml) and DAPI. (1μg/ml) to give banded chromosomes which were used for chromosome identification: digital images were produced using a Bio Rad laser scanning confocal microscope.

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REFERENCES

1. Moroi, Y., Peebles, C., Fitzler, M.J., Steigerwald, J. and Tan, E.M (1980) *Proc. Natl. Acad. Sci* USA, 77, 1627–1631.
2. Willard, H.F.(1990) *Trends in Genetics*, 6, 410–416.
3. Earnshaw, W.C., Rattie H. and Sutton, G. (1989) *Chromosoma*, 98, 1–12.
4. Sintoh, H., Tomkiel, J., Cooke, C.A., Rattie, H.,III. Maurer, M . Rothfield N.F. and Earnshaw W.C. (1992) *Cell* 70, 115–125.
5. Hadlaczky, G., Praznovszky, T., Csorpan, I., Kereso, J., Peterfy, M.; Kelemen, I., Atalay E., Szeles, A.; Szelei, J., Tubak, V., (1991) *Proc. Natl.Acad Sci U.S.A.*, 88, 8106–8110.
6. Clarke, L. (1990) *Trends in Genetics* 6 150–154.
7. Haaf, T., Warburton, P., and Willard, H.F (1992) *Cell* 70 681–696.
8. Maniatis, T., Fritsch, G. F. and Sumbrook, J. (1982) Molecular cloning: A Laboratory Manual. Cold Spring Harbour Press.
9. Jones, C. and Kao F.T. (1983) *Cytogenet. Cell Genet.* 37 499–500.
10. Laird P. W., Zijderveld, A., Linders, K., Rudnicki, M., Jaenisch, R., Berns, A. (1991) *Nucleic Acids Res.*, 19, 4293 4297.
11. Church,G.M. &Gilbert,W. (1984) *Proc.Natl.Acad.Sci. U.S.A.*, 81,1991–1995.
12. Lichter, P., Chieh-ju, C. T., Call, K., Hermanson, G., Evans G. A., Housman, D. and Ward, D. C. (1990) *Science* 247, 64–69.

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The putative centromere-forming sequence λCM8 is a single copy sequence and is not a component of most human centromeres

Katrina F.Cooper and Chris Tyler-Smith*

CRC Chromosome Molecular Biology Group, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

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The DNA sequences which form a human centromere are unknown. Structural analysis has shown that these regions contain long arrays of several classes of tandemly repeated 'satellite' sequences. One of these, alphoid satellite DNA, has been found to have some of the properties of a centromere when introduced into African Green Monkey cells (1). In addition, the human sequence λCM8, which is unrelated to the known satellites, was reported to form a centromere after transformation into mouse cells (2). The copy number of λCM8 in the human genome was estimated to be 16–32 and it was found by *in situ* hybridisation experiments to be present at the centromere of many human chromosomes (2). It therefore seemed possible that λCM8 could be an essential functional component of human centromeres, and was perhaps located near the satellite DNA. We are carrying out an analysis of the structure of the human Y chromosome centromere, and therefore set out to place λCM8-related sequences in the Y chromosome map.

In initial experiments using six subclones spanning λCM8 (pCM1, pCM6, pCM11, pCM16, pCM21 and pK1620) we detected no hybridisation to the Y chromosomes in three different 'Y only' somatic cell hybrids. In addition, the intensity of hybridisation to human DNA was less than expected for a 16–32 copy sequence.

We therefore measured the copy number of λCM8-related sequences in human DNA (Figure 1). An equimolar mix of a subclone of λCM8 (pCM11) and a known single-copy sequence (pMC1.4) located ~40kb upstream from the α globin genes on chromosome 16 (3) was prepared. A sample of the mix was fractionated by gel electrophoresis and transferred to a filter. Hybridisation to a ^{32}P-labelled sample of the same mix followed by phosphorimager quantitation showed approximately equal levels of hybridisation in the two samples: pCM11, 5014 units; the single copy probe pMC1.4, 4521 units. The mixture was then hybridised to *Hin*dIII digests of human, Y hybrid and revertant DNA samples. Figure 1 shows that in human DNA the intensity of hybridisation to pCM11 (eg. 5124 phosphorimager units in the male track) is similar to the intensity of hybridisation to the single copy probe pMC1.4 (5875 units in the male track). When allowance is made for the small differences in concentration and/or labelling efficiencies of the two probes, the copy number ratio of pCM11: pMC1.4 is 0.8:1. As expected, the α globin region probe is not present on the Y chromosome, and it can be seen that pCM11 is also not present on the Y chromosome. The similarity in the level of hybridisation to male and female DNA also excludes pCM11 from the X chromosome.

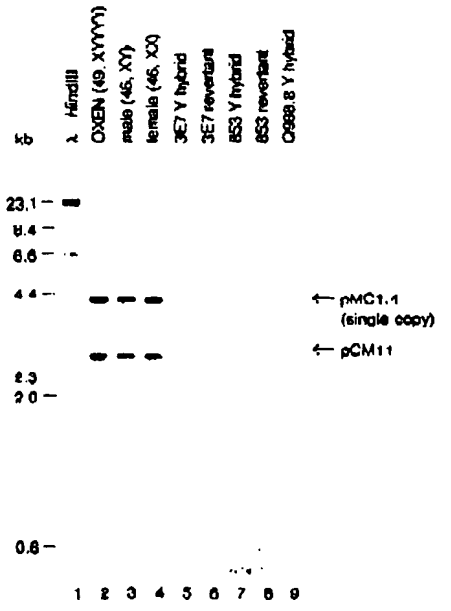

Figure 1. Measurement of the copy number of pCM11. DNA samples (as indicated at the top of each track) were probed with an equimolar mixture of pCM11 and the single-copy sequence pMC1.4. Experimental methods were as described (4). Fragment sizes in kilobases (kb) are indicated on the left-hand side.

These results show that λCM8 is an autosomal single copy sequence in the human genome and contradict the previous suggestion that it is present in 16–32 copies and is located at the centromere of multiple human chromosomes (2). Experimental data supporting the higher copy number were not given and so cannot be assessed. *In situ* hybridisation was not detected when biotin-labeled λCM8 was used; [^{3}H] thymidine-labeled λCM8 showed only about a three-fold enrichment of silver grains in the centromeric regions (2) and this could represent non-specific binding. It therefore seems likely that the original characterisation was erroneous. λCM8 cannot be a component of most centromeres, and therefore cannot be required for centromere function. Its ability to form a centromere after transformation into mouse cells is thus puzzling. Two explanations can be given. It is possible that the observed

* To whom correspondence should be addressed

centromeric activity was due to the incorporation of mouse centromeric sequences (e.g. mouse satellite DNAs) with the transfected DNA and thus that λCM8 has no intrinsic centromere-forming properties. Alternatively, the sequence requirements for a centromere could be rather non specific, and could perhaps be satisfied by many sequences when they are present in multiple copies. If, for example, λCM8 contains a protein binding site, then one copy in its normal location might have no centromeric activity but the large number of copies which are produced during transformation might bind sufficient protein to form a centromere *de novo*. Additional transformation experiments using λCM8 and unrelated sequences could test these hypotheses.

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REFERENCES

1. Hsu,T., Warburton,P.E. and Willard,H.F. (1992) *Cell* 70, 681–696.
2. Hadlaczky,G., Praznovszky,T., Cserpán,I., Keresö,J., Péterfy,M., Kelemen,I., Ataluy,E., Szeles,A., Szelei,J., Tubak,V. and Burg,K. (1991) *Proc. Natl. Acad. Sci. USA* 88, 8106–8110.
3. Higgs,D.R., Wood,W.G., Jarman,A.P., Sharpe,J., Lida,J., Pretorius,I.-M. and Ayyub,H. (1990) *Genes Dev.* 4, 1588–1601.
4. Cooper,K.F., Fisher,R.B. and Tyler-Smith,C. (1992) *J. Mol. Biol.* 228, in press.